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## WIDELY DISTRIBUTED NECROTIZING ARTERITIS INDUCED IN RABBITS BY EXPERIMENTAL RENAL ALTERATIONS

### I. COMPARISON WITH THE VASCULAR LESIONS INDUCED BY INJECTIONS OF FOREIGN SERUM \*

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Hypertension is a frequent consequence of certain experimental renal alterations induced in animals, principally rats. These alterations are also often followed by widely distributed necrotizing vascular lesions which have been considered by many to resemble those of periarteritis nodosa in man.<sup>1-10</sup> Hypertension, particularly of rapidly rising character, has been believed to be the causative factor in the pathogenesis of these experimental lesions.<sup>1,2,5,6,10</sup> On the other hand, other investigators have induced vascular lesions in rabbits by one or more parenteral injections of foreign protein. These, too, have been considered to closely resemble periarteritis nodosa, and have been attributed to hypersensitivity.<sup>11-21</sup> Obvious variables are inherent in the use of two species (rats and rabbits) in a comparative study of vascular lesions induced by two different experimental methods. It seemed important to utilize a single species of animal to inquire in parallel into the pathogenesis of vascular lesions (a) associated with renal alterations and hypertension, and (b) induced by injections of foreign protein.

In the present investigation, widely distributed arteritis has been induced consistently in rabbits by unilateral perinephritis followed in 7 days by contralateral nephrectomy. The latter procedure was regularly followed by hypertension. In another group of rabbits, arteritis was induced by the injection of foreign serum. The results of a comparative histologic study of these two sets of arterial lesions are here reported.

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## METHODS

*Group I. Induction of Lesions by Unilateral Perinephritis  
Followed by Contralateral Nephrectomy*

Seventeen rabbits of both sexes, 4 to 12 months old, were used. Fifteen of these animals were from the stock of the Rockefeller Institute for Medical Research, New York City. Among rabbits of this stock, spontaneous interstitial myocarditis and lesions caused by *Coccidium perforans* occurred very rarely. Moreover, in the course of microscopic examination of sections from many hundreds of rabbits of this stock during the past 10 years, neither spontaneous interstitial pyelonephritis nor spontaneous necrotizing vascular lesions have been encountered.<sup>22</sup>

By a posterior retroperitoneal approach under strict aseptic conditions, the left kidney was reached and snugly wrapped in silk. Thin white Japanese silk was cut in the shape of a butterfly. A hole approximately 5 mm. in diameter was made in its center, and into this the hilar structures fitted with ease. The "wings" of the preparation enclosed the kidney and were sutured together along its lateral border. Steam distilled turpentine (3 ml., Alpha brand, from George Isaacs and Company, New York City and South Kearney, New Jersey) was then dropped onto the wrapped kidney, and the organ was returned to its original position. Right nephrectomy was performed 7 days later. Dating from the induction of the silk-and-turpentine perinephritis, the rabbits died or were sacrificed as follows: 1 rabbit at 10 days; 2 rabbits at 15 days; 4 rabbits at 22 days; 3 rabbits at 33 days; 3 rabbits at 44 days; 1 rabbit at 53 days; 1 rabbit at 106 days; 1 rabbit at 184 days; and 1 rabbit at 908 days.

Blood pressure measurements were made each day or every other day on all rabbits in this group.

*Group II. Induction of Lesions by Multiple Injections of  
Foreign Serum*

The method utilized was essentially that employed by Rich and Gregory.<sup>14</sup> Thirteen rabbits of both sexes, 4 to 12 months old, were utilized. Ten of these animals were from the stock of the Rockefeller Institute noted above. Sterile, fresh horse serum without preservative (supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York) was used for injection. The rabbits received 10 ml. of horse serum per kg. of weight intravenously on day zero. On the twelfth day, all rabbits received intradermal injections of horse serum (0.1 ml.) and subsequently developed



indurated, erythematous reactions. On the 15th day, 1 ml. of horse serum was slowly injected intravenously into each rabbit. On the 17th day, a second large dose of horse serum (10 ml. per kg. of weight) was administered intravenously. Dating from the administration of the second large dose of serum, rabbits were sacrificed as follows: 3 rabbits at 3 days; 4 rabbits at 9 days; 3 rabbits at 16 days; and 3 rabbits at 31 days.

Blood pressure measurements were made each day or every other day on the rabbits sacrificed on the third and 31st days.

#### *Blood Pressure Determination*

A modification of the Grant-Rothschild capsule was utilized in the determination of blood pressure.<sup>23</sup> The apparatus consisted of a small electric light source mounted behind a translucent plastic capsule. A thin, translucent, polyvinyl membrane covered the capsule. The apparatus measures the pressure in millimeters of mercury necessary to occlude the central ear artery. This value is a function of the systolic blood pressure of the carotid and femoral arteries<sup>23,24</sup> and correlates with values for systolic blood pressure obtained by direct cannulation of the central artery of the opposite ear.<sup>25</sup> On each day that the blood pressure was measured, the values of 4 readings, usually 2 from each ear, were obtained. The individual readings were in the range of plus or minus 7 mm. of mercury from the daily average. The 8 values for blood pressure of 2 consecutive days were averaged and graphed.

#### *Cardiac Index Determination*

The carcass weight is here defined as the weight of the rabbit after necropsy and removal of brain, pituitary, lungs, heart, thymus, spleen, pancreas, gonads, aorta, alimentary tract, and small blocks of other tissues. The cardiac index is defined by the formula,  $\frac{\text{heart weight}}{\text{carcass weight}}$ . Pickering and Prinzmetal<sup>24</sup> have emphasized that a significant increase in this ratio is a sensitive index of cardiac hypertrophy. In a series of 32 normal rabbits, these investigators found the cardiac index to have a mean value of  $2.5 \times 10^{-3}$  (range 2.0 to  $3.3 \times 10^{-3}$ ).

#### *Histologic Procedures*

Microscopic examination was regularly carried out on the following tissues: heart, lung, spleen, pancreas, liver, kidney, adrenal, testis or ovary, aorta, mesentery, thymus, diaphragm, and other voluntary muscles. In 22 rabbits, the following organs were also examined: esophagus, stomach, small intestine, cecum, appendix, large intestine,

thyroid and brain. Sections of skin, lymph node, gall bladder, fallopian tube and salivary gland were available in several animals. Brain, pituitary, and selected tissues for special studies were fixed in 10 per cent formalin-bromide solution; all other blocks were fixed in Zenker's solution. Paraffin sections were routinely stained with hematoxylin and eosin. Selected sections were stained with Giemsa, Masson's trichrome, Weigert-van Gieson, Weigert-hematoxylin, phosphotungstic acid-hematoxylin, and periodic acid-Schiff stains.

### RESULTS

Hypertension invariably developed in rabbits in group I (unilateral silk-and-turpentine perinephritis and contralateral nephrectomy; Text-figure 1). On the other hand, the blood pressure was measured in 6 of the 13 rabbits in group II (multiple injections of horse serum); none developed hypertension. In fact, the blood pressure in the latter animals fell slightly below the range of normal during the interval between the 14th and 16th experimental days. It remained at this lower level for about 2 weeks and then returned to about the lower limit of normal (Text-fig. 1). The fall in blood pressure occurred during the period in which other investigators<sup>17,21,26</sup> had shown that foreign protein antigens were being cleared from the blood and the titer of circulating antibodies was rising. In our opinion, this interval of relative hypotension might be related to the development of cardiac lesions, particularly in the myocardium.

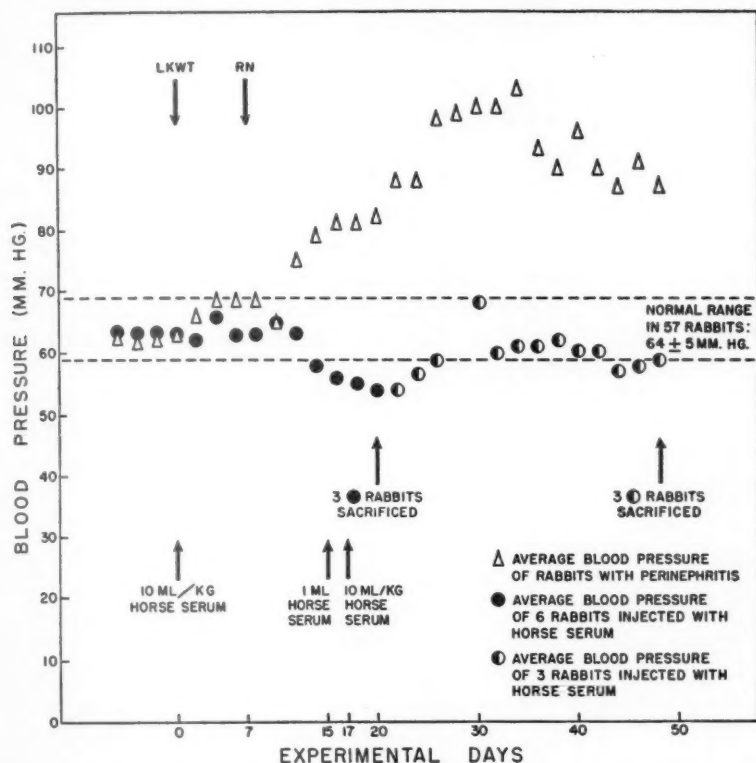
Hopps and McCollum,<sup>28</sup> Smull, Wissler and Watson,<sup>29</sup> and Orbison<sup>30</sup> found no significant rise in blood pressure in rabbits receiving injections of foreign protein. Smull and co-workers<sup>29</sup> reported a fall following a second large injection of horse serum. Germuth and Heptinstall<sup>31</sup> observed no blood pressure rise in 25 rabbits that received multiple injections of bovine gamma globulin. A rise in blood pressure was encountered in one rabbit which developed acute glomerulonephritis.

#### *Morphologic Observations*

*Kidney.* The silk-wrapped kidneys of the rabbits in group I had distinctly pale cortices.<sup>27</sup> Neither renal infarcts nor obstructive lesions of the urinary tract were encountered in this group. The perirenal structures were the seat of varying degrees of inflammation and fibrosis. On the other hand, the kidneys of the rabbits in group II were grossly normal.

*Cardiovascular System.* Lesions encountered in the two experimental groups differed significantly (Table I). In group I numerous petechiae were scattered in the alimentary tract and thymus of all

rabbits that died or were sacrificed 15 to 53 days after the induction of perinephritis. Hemorrhages in the brain were observed in 5 rabbits of this group (Figs. 13 and 14). In one of these there was also an



Text-figure 1. Changes in blood pressure in Group I rabbits; left kidney wrapped with turpentine-saturated silk (LKWT), followed in 7 days by right nephrectomy (RN). These are contrasted with blood pressure readings in group II rabbits which received injections of horse serum. Rabbits with perinephritis were sacrificed at different time intervals, and graphed values of blood pressure are an average from 7 or more animals. Note that the blood pressure began to rise about 5 days after nephrectomy and progressed to a peak at 33 days.<sup>27</sup> In sharp contrast, the blood pressure in serum-treated animals fell slightly below the range of normal on the 14th day, i.e., one day before the administration of the second intravenous injection of serum (1.0 ml.). The blood pressure remained depressed in 3 animals for about 2 weeks and then returned to about the lower limit of normal.

infarct in the right occipital lobe (Fig. 14). Rare subepicardial petechiae were the only hemorrhagic phenomena encountered in the rabbits of group II, and were seen in 2 of 13.

The cardiac indices of the rabbits in group I ranged from 2.88 to  $4.47 \times 10^{-3}$ . This range was higher than the values obtained in con-

TABLE I  
*Morphologic Observations*

| Group I*  | Group II*   |
|---|---|
| <p>1. Small muscular arteries and arterioles involved. Veins spared. Most lesions in G.I. tract, liver, gall bladder. Diaphragm, brain, gonads, heart also affected. Lung, pancreas, spleen and kidney unaffected.</p> <p>(a) Early arterial lesion 15 to 22 days after perinephritis. Circumferential involvement. Endothelial pyknosis; medial necrosis with fibrin-like staining; adventitial infiltration by histiocytes, lymphocytes and neutrophils.</p> <p>(b) Late arterial lesions 33 or more days after perinephritis. Cellular, circumferential intimal thickening; thinning of media; concentric reduplication of internal elastic membrane; adventitial healing by concentric collagenous bands; lumen round or oval.</p> <p>2. Widely distributed petechial hemorrhages, especially in G.I. tract; hemorrhage in brain in 5 of 13 rabbits.</p> <p>3. Cardiac index elevated.</p> <p>4. Myocardium normal.</p> <p>5. Proximal aorta normal.</p> <p>6. Valvular endocardium normal.</p> <p>7. Wrapped kidneys distinctly pale.</p> <p>8. No pericardial effusion.</p> <p>9. Low grade ascites common.</p> <p>10. Normal thymus, spleen and lymph nodes.</p> | <p>1. Arteries of all sizes, arterioles, and veins involved. Most lesions in heart; fewer in lung, kidney, and large branches of aorta.</p> <p>(a) Early arterial lesion 3 days after second large injection of serum. Segmental; swollen, proliferated subendothelium; swelling and degeneration of media (cytolysis); adventitial infiltration by plasma cells, lymphocytes and histiocytes.</p> <p>(b) Late arterial lesions 9 or more days after second large injection of serum. Nodular; segmental fibrocellular thickening of intima; nonconcentric proliferation of internal elastic membrane. Adventitia segmentally scarred. Slit-like lumen.</p> <p>2. Rare petechial hemorrhages; no cerebral hemorrhage.</p> <p>3. Cardiac index normal.</p> <p>4. Myocarditis, with or without necrosis or scarring.</p> <p>5. Aortitis, proximal aorta.</p> <p>6. Valvular endocarditis.</p> <p>7. Kidneys normal in gross.</p> <p>8. Pericardial effusion common.</p> <p>9. No ascites.</p> <p>10. Hyperplasia of thymus, spleen and lymph nodes.</p> |

\* Group I: Rabbits in which unilateral perinephritis was induced by wrapping kidney with turpentine-soaked silk followed by contralateral nephrectomy.

Group II: Rabbits receiving multiple injections of horse serum.

trol rabbits of the same age, weight and stock.<sup>24,27</sup> In serum-treated animals of group II, the cardiac indices remained within normal range;  $2.39$  to  $3.18 \times 10^{-3}$ .

In all 13 rabbits of group II, focal myocardial lesions characterized by myocarditis (with or without necrosis) or scarring were observed; focal valvular endocarditis appeared in 8 of these animals. These lesions have been observed by others<sup>12-21</sup> in animals receiving injections of foreign protein. We found no cardiac lesions of this nature in animals with perinephritis (group I).

The distribution of the vascular lesions in the two experimental groups differed significantly (Table I).

Microscopically, all animals in group I (except one dying on the tenth day) exhibited lesions only in the small arteries and arterioles. These were found with regularity in the alimentary tract, liver, gall bladder, and diaphragm; alterations in the small coronary arteries were infrequent. Although arterial and arteriolar lesions were widely distributed in most animals, they were not encountered in the lung, kidney, pancreas, or spleen. In 2 of 8 rabbits with silk-and-bacterial perinephritis and contralateral nephrectomy, Kipkie<sup>7</sup> noted involvement of the small arteries in the alimentary tract, liver, pancreas, salivary gland, and skeletal muscle.

In sharp contrast, the rabbits of group II with experimental serum disease exhibited lesions in arteries of all sizes in both heart and lung; those in the coronary vessels were particularly numerous. Medium and small sized pulmonary veins were also affected. About half of the animals showed one or two lesions in the large branches of the celiac axis and/or superior and inferior mesenteric arteries. Some of the smaller renal arteries and arterioles were cuffed by lymphocytes and plasma cells. Although a few pulmonary, renal, and larger splanchnic vessels were affected in this group, the total number of vascular lesions found outside the heart in each animal was very small. These observations are in agreement with those of other investigators.<sup>18,19</sup>

Histogenetically, the vascular lesions associated with perinephritis (group I) on the one hand and those induced by the injections of horse serum (group II) on the other differed significantly (Table I). The characteristic early arterial alterations in animals with perinephritis was coagulative necrosis of the entire media. The lesion was hypereosinophilic and had staining qualities resembling those of fibrin with phosphotungstic acid-hematoxylin, Masson's trichrome and periodic acid-Schiff stains. It was bright yellow with the Weigert-van Gieson stain. In a few instances, focal aneurysmal dilatation of small

necrotic arteries was evident (Figs. 3 and 4). Pyknosis of the endothelial nuclei was often found (Fig. 1). The early lesions in rabbits given serum usually involved only part of the circumference of a vessel (Fig. 17), whereas those associated with perinephritis were usually completely circumferential (Figs. 2 and 4).

Characteristically, the early lesions in animals of group II exhibited edema of the intima with some degeneration of the subintimal structures. The subjacent media was the seat of swelling and some degeneration of the smooth muscle fibers (Figs. 16 and 18). The term "cytolysis" perhaps best describes these changes. Small patchy areas which stained like fibrin were found infrequently in the adventitia and even less often in the media (Figs. 17 and 19). In severely affected arteries there were focal, proliferative, intimal lesions, and the media showed disoriented, edematous smooth muscle, binucleated cells and small inflammatory cells (Fig. 20). Rich and Gregory<sup>14</sup> pointed out that the early lesion of serum disease was characterized by a sharply localized segmental distention of the arterial wall. This was the result of localized edema, having the appearance of an urticarial wheal. Germuth<sup>20</sup> likewise noted that only a portion of the circumference of a medium-sized artery usually showed evidence of injury. When the blood vessel involved was small, however, often its entire circumference was injured. The early adventitial cellular reaction in rabbits given serum was usually focal. In the animals with perinephritis it was usually distributed circumferentially. Neutrophils appeared more frequently in the adventitial reaction in group I (Fig. 2); lymphocytes and plasma cells were more common in group II (Figs. 16 and 22).

Eleven of the 13 rabbits that received serum had inflammation of the first 2 cm. of the aorta. The aortitis was characterized by collections of lymphocytes and plasma cells in the intima; the lesion was not observed in the animals of group I.

The late stages in the two groups also differed significantly. In group I, healing lesions in one coat of a vessel were often accompanied by early alterations in other coats. In particular, early intimal and medial lesions were frequently associated with organization of granulation tissue in the adventitia. The latter appeared as concentric rings of histiocytes, fibroblasts, and collagen (Figs. 5 to 7); the concentric pattern persisted in the healed, scarred state. Medial and intimal sclerosis also had a concentric arrangement, and concentric reduplication of the internal elastic lamina was not uncommon (Figs. 8 to 12).

On the other hand, in rabbits of group II the healing lesions at the



same stage of development involved all coats of the blood vessel wall. Intimal proliferation was frankly segmental and usually resulted in marked slit-like narrowing of the lumen of the vessel. There was little edema or necrosis in the subendothelial tissues (Figs. 21 and 22). The smooth muscle of the media was replaced focally by binucleated cells and proliferating smaller elements with vesicular nuclei (Fig. 21). Inflammatory reaction in the adventitia was a less prominent feature. The healed arterial lesion in this group showed nodular, segmental scarring. The internal elastic lamina was infrequently interrupted; it was often reduplicated but not in completely concentric rings (Figs. 23 and 24).

The necrotizing arterial lesions and the accompanying increased blood pressure in group I probably contributed to aneurysmal dilatation, rupture of affected vessels and hemorrhage (Figs. 3, 4 and 13). The focal, proliferative arterial lesions (Figs. 16 to 18, and 20 to 22) in rabbits of group II were unaccompanied by hypertension and were complicated by neither aneurysm formation nor hemorrhage.

#### DISCUSSION

Widely distributed arteritis was consistently induced in rabbits by wrapping one kidney with turpentine-saturated silk and following this in one week by contralateral nephrectomy. The histogenesis of the lesions, contrasted with that characterizing the vasculitis induced in rabbits by injections of foreign serum, have clearly demonstrated a difference between the two forms of necrotizing arteritis (Table I).

Masson, Hazard, Corcoran and Page<sup>6</sup> carried out an extensive comparative study in rats of vascular lesions associated with the production of silk-perinephritis, and with the administration of deoxycorticosterone and anterior pituitary extracts. These investigators reported that the arterial lesions induced by these 3 techniques could not be differentiated histologically. However, it is noteworthy that the kidney was nearly always the only site of arterial lesions when anterior pituitary extract was administered, whereas arterial lesions were widely distributed when the two other techniques were employed. On the other hand, Murphy and Swift<sup>32</sup> showed that vascular lesions induced in rabbits by repeated focal infections with group A streptococci differed significantly from those in rabbits with experimental serum disease. In parallel, they also pointed out numerous differences between the vascular lesions of rheumatic fever and those of human serum disease. Kolff and Fisher<sup>33</sup> stated that morphologic differences existed between the arterial lesions induced by bilateral nephrectomy

and those caused by the administration of deoxycorticosterone in the rat.

Zeek and associates<sup>5,34,35</sup> have classified human necrotizing angiitis into 5 categories: hypersensitivity angiitis, allergic granulomatous angiitis, rheumatic arteritis, periarteritis nodosa and temporal arteritis. On the other hand, some investigators have expressed the opinion that the morphologic differences between "hypersensitivity angiitis" and "classical periarteritis nodosa" noted by Zeek and associates in man are not unlike those observed in rabbits that have been exposed to antigenic material in acute and chronic experiments respectively.<sup>20,36</sup>

The present experiments have demonstrated that in one species, the rabbit, significantly different patterns of vascular lesions have been induced in the course of experimental serum disease and in association with unilateral silk-and-turpentine perinephritis and contralateral nephrectomy. Various vascular beds and varying sizes and types of blood vessels have been shown to respond differently in these two experimental conditions.

#### SUMMARY

A morphologic comparison was made between the vascular lesions induced in rabbits of two groups. In one (group I) the left kidney was wrapped in silk saturated with turpentine. This was followed in one week by contralateral nephrectomy. Rabbits of the other group (group II) received multiple injections of horse serum. The rabbits with perinephritis developed hypertension following contralateral nephrectomy. Hypertension did not develop in the rabbits receiving horse serum.

There were distinct morphologic differences in the vascular lesions that developed in the two groups. The lesions were distinguishable by (a) the size and type of vessel affected; (b) the distribution of the lesions; and (c) the distinctive histologic characteristics in the various stages of development.

Lesions of small muscular arteries and arterioles in rabbits of group I were found to be widely distributed and especially numerous in the alimentary tract, liver, gall bladder, and diaphragm. They were less common in the brain, gonads, and heart. Early alterations were characterized principally by circumferential coagulative necrosis of the media and the appearance of a substance with fibrin-like staining quality. Late arterial lesions typically comprised necrosis in one coat and concentric healing in another coat of a vessel wall or concentric scars in all coats. In sharp contrast, in rabbits of group II arteries of all sizes, arterioles, and veins were affected. The lesions here were

numerous in the heart; a few were also found in the lung, kidney, and larger branches of the aorta. The early alterations were characterized by segmental edema of the subendothelial tissue and cytolysis of the subjacent media. These were followed by marked cellular intimal proliferation, also with segmental distribution. An early lesion in one coat was rarely accompanied by a late one in another coat of the same vessel. The late lesions were usually characterized by focal healing or scarring of the vessel wall.

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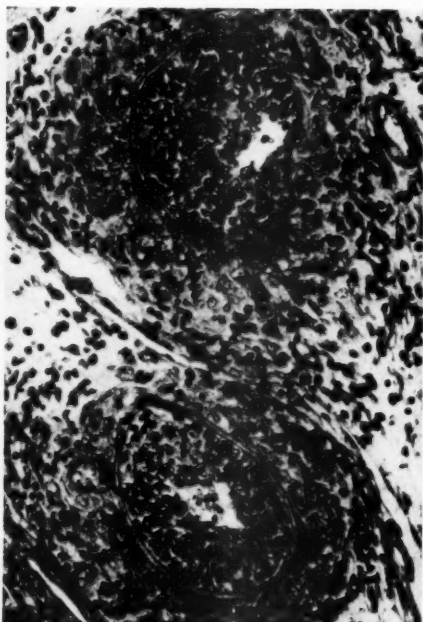
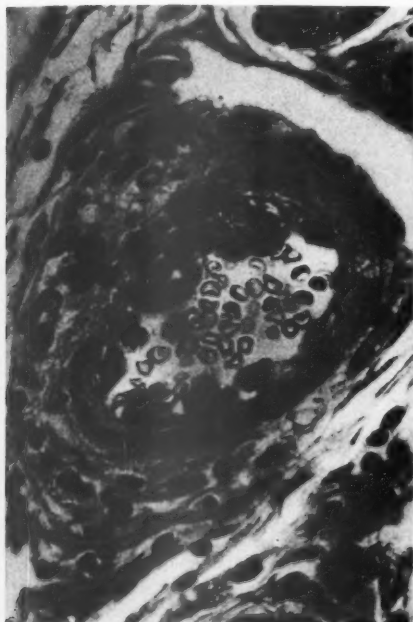
[ Illustrations follow ]

## LEGENDS FOR FIGURES

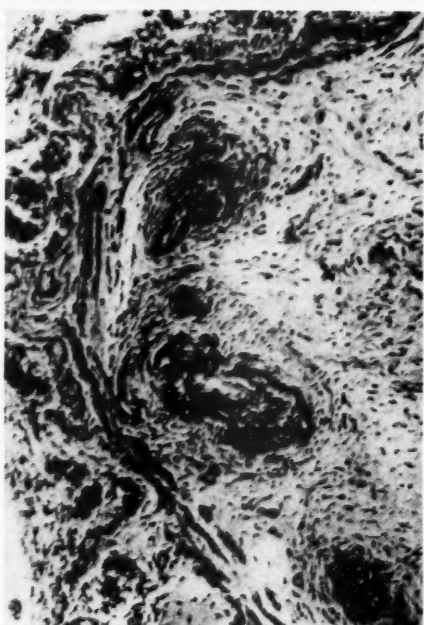
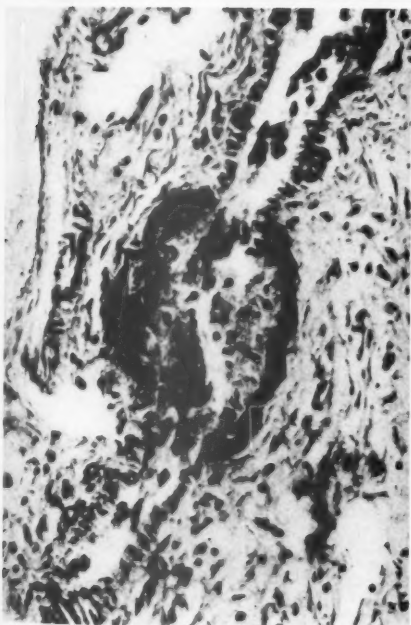
Figures 1 through 15 illustrate vascular lesions in group I rabbits (left kidney wrapped in turpentine-saturated silk, right kidney removed 7 days later). Figures 16 through 24 illustrate lesions in group II rabbits sacrificed after multiple injections of horse serum.

- FIG. 1. An early lesion in a small arteriole in the duodenal submucosa. Rabbit sacrificed 22 days after induction of perinephritis. Endothelial cells are rounded, and their nuclei and those of some of the smooth muscle cells are pyknotic. There is slight cellular infiltration by lymphocytes and histiocytes in the adventitia and adjacent damaged endothelium. The internal elastic lamina is fragmented at one o'clock. Hematoxylin and eosin stain.  $\times 677$ .
- FIG. 2. Well developed but relatively early lesions in two small arteries in the colonic submucosa 15 days after the induction of perinephritis. There is marked cellular reaction, and vessel walls are essentially destroyed except for parts of the internal elastic laminae. The latter are thickened and have lost their wavy appearance. Hematoxylin and eosin stain.  $\times 284$ .
- FIG. 3. Longitudinal section of a small artery in the duodenum of a rabbit which died of massive brain stem hemorrhage 33 days after the induction of perinephritis. There is aneurysmal dilatation of the focally necrotic arterial wall. The dark area represents a necrotic zone which stains in fibrin-like fashion. Phosphotungstic acid-hematoxylin stain; restained with Giemsa stain.  $\times 165$ .
- FIG. 4. Three small arteries in the submucosa of the gall bladder. Rabbit sacrificed 15 days after the induction of perinephritis. There is coagulative necrosis, represented by dark staining in the dilated wall of all 3 vessels. Masson's trichrome stain.  $\times 132$ .





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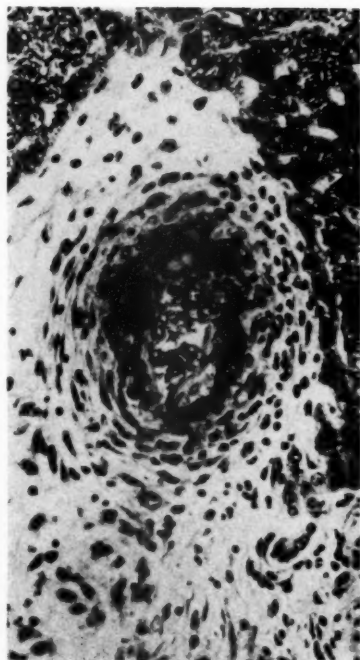
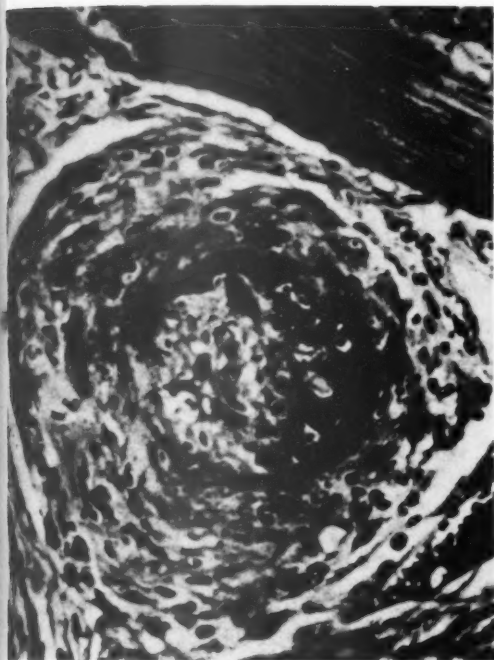


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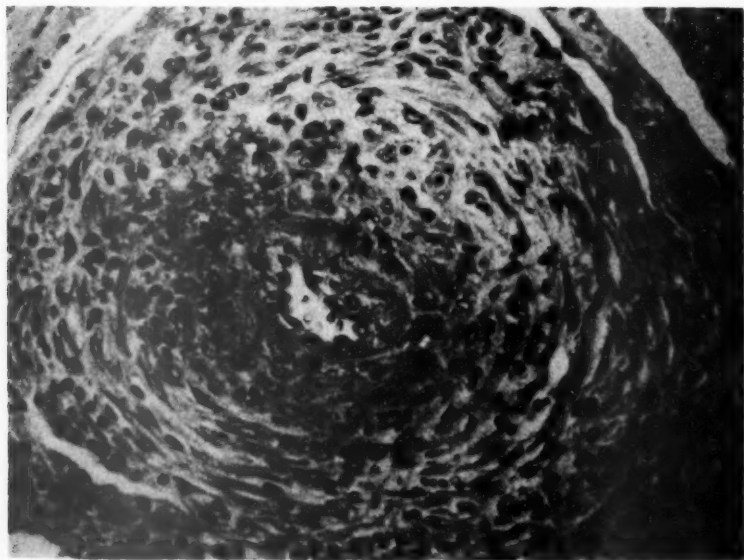
FIG. 5. Small artery in the small intestine of a rabbit sacrificed 34 days after the induction of perinephritis. This is a healing lesion, characterized by an organizing reaction in the adventitia. Round cells and fibroblasts surround the vessel in concentric fashion. The lumen is nearly occluded by an amorphous, stringy substance which also surrounds intimal cells. A large portion of the media stains in fibrin-like manner. Masson's trichrome stain.  $\times 422$ .

FIG. 6. Small artery in a portal area of the liver of a rabbit sacrificed 43 days after the induction of perinephritis. This is a healing lesion with concentrically arranged bands of histiocytes, fibroblasts, and collagen in the adventitia. Coagulative necrosis is evident in the subendothelial region and media, and much of this stains in fibrin-like fashion. Phosphotungstic acid-hematoxylin stain.  $\times 426$ .

FIG. 7. Submucosal artery in the jejunum of the rabbit from which Figure 1 was obtained. A healing lesion with concentric rings of fibroblasts, collagen and histiocytes appears in the adventitia. On the other hand, there is also necrosis in the subendothelial region and media, indicative of recent damage. Hematoxylin and eosin stain.  $\times 377$ .

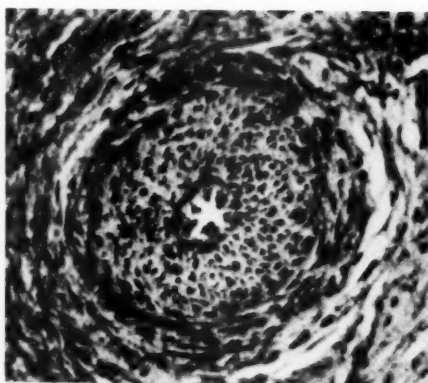
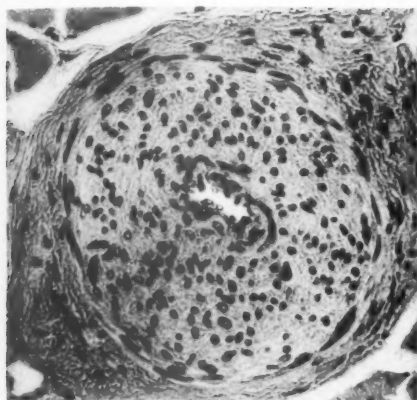


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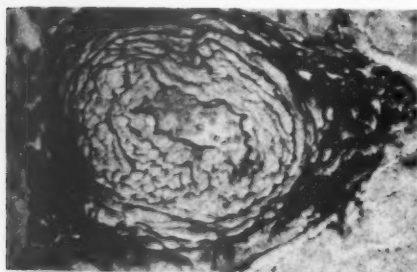


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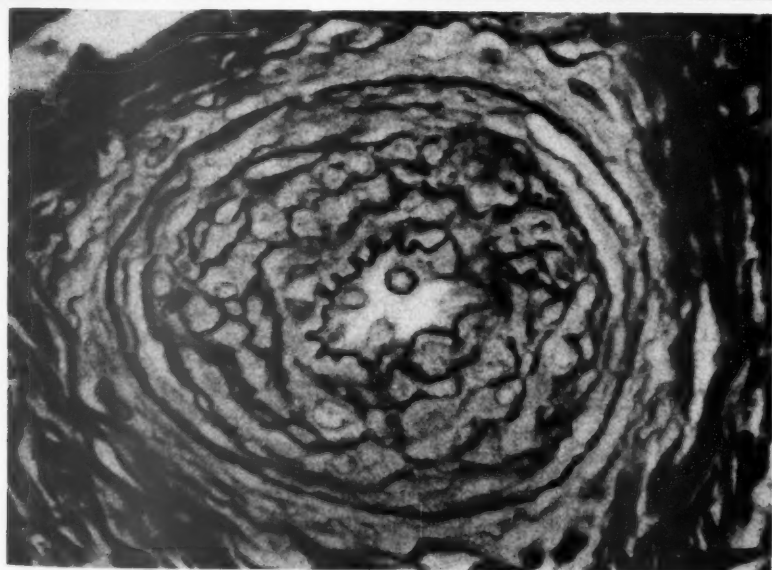
- FIG. 8. Small artery in the diaphragm of a rabbit sacrificed 44 days after the induction of perinephritis. In this healed lesion the lumen is narrowed by circumferential cellular thickening of the intima. The media is reduced to one or two layers of smooth muscle elements with elongated nuclei. Hematoxylin and eosin stain.  $\times 366$ .
- FIG. 9. Small hepatic artery in the liver of the rabbit from which Figure 3 was obtained. A healed lesion, showing cellular intimal thickening and a markedly thinned media. Portions of the media and adventitia are replaced by concentric bands of collagen. Masson's trichrome stain.  $\times 179$ .
- FIG. 10. Small coronary artery in the left ventricle of a rabbit sacrificed 106 days after the induction of perinephritis. A healed lesion, characterized by two complete internal elastic laminae, one beneath the endothelium and the other between the intima and media. Weigert-van Gieson stain.  $\times 140$ .
- FIG. 11. An artery as it enters the small intestine in the rabbit from which Figures 3 and 9 were procured. In this healed lesion thrombosis of the lumen and organization have taken place. The internal elastic lamina is not reduplicated, and the media and adventitia are thickly replaced by many concentric bands of collagen, imparting an "onion skin" appearance. Weigert-van Gieson stain.  $\times 125$ .
- FIG. 12. Small submucosal artery in the small intestine of a rabbit sacrificed 44 days after the induction of perinephritis. This healed lesion exhibits marked symmetrical narrowing of the lumen by subendothelial cells with round nuclei. The internal elastic lamina is thinned, beaded and concentrically reduplicated. There is little interlacing of elastic fibers. The media is thinned to a single layer of cells. Weigert-van Gieson stain.  $\times 1,065$ .



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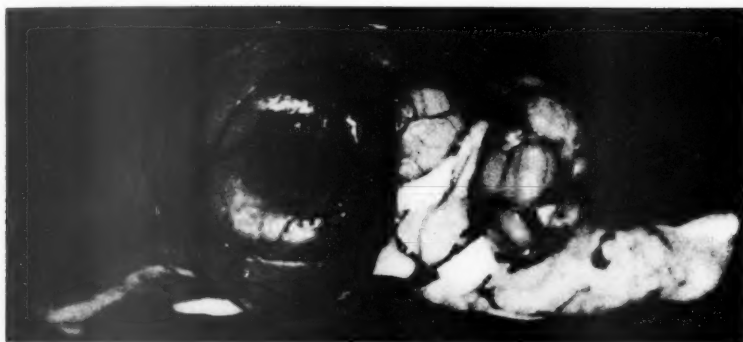
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- FIG. 13. Brain of a rabbit sacrificed 43 days after the induction of perinephritis. A large recent subarachnoid hemorrhage and several adjacent petechiae appear over the lateral aspect of the left occipital lobe.  $\times 2$ .
- FIG. 14. Brain of rabbit from which Figure 5 was procured. There is hemorrhagic softening of the right occipital lobe. Petechial hemorrhages are scattered along the courses of small arteries.  $\times 2$ .
- FIG. 15. A small leptomenigeal artery on the surface of the brain of the rabbit from which Figure 12 was procured. This is an early lesion with pyknosis of endothelial and muscle nuclei. Eosinophilic substance is evident beneath the endothelium. Histiocytes and lymphocytes infiltrate the vessel wall. Hematoxylin and eosin stain.  $\times 433$ .

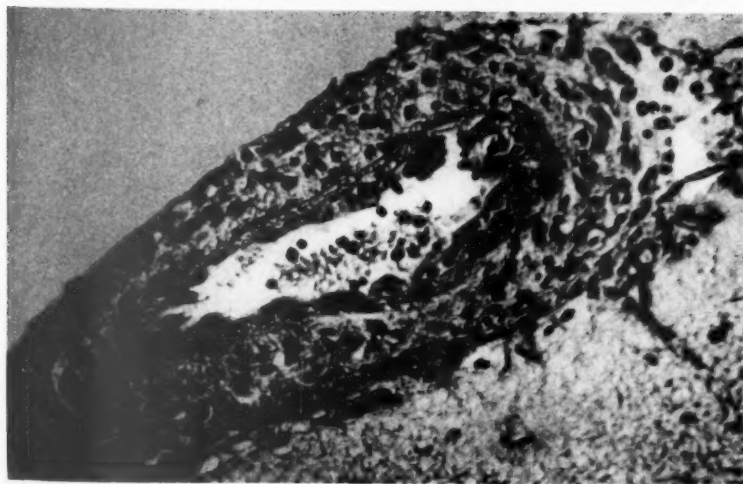




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FIG. 16. A large coronary artery in the left ventricle of a rabbit sacrificed 3 days after a second large injection of horse serum. Focal subendothelial swelling such as that at 9 o'clock characterizes the earliest alteration encountered. Another segmental lesion is evident in the lower portion of the photograph. Visible from 3 to 7 o'clock are subendothelial edema and medial necrosis (cytolysis). This portion of the wall is infiltrated by lymphocytes and histiocytes. Hematoxylin and eosin stain.  $\times 138$ .

FIG. 17. A large branch of the left coronary artery. Same heart illustrated in Figure 16. Lesion is characterized by segmental, subendothelial edema and necrosis (cytolysis) of the media. The dark region in the adventitia on the left exhibits fibrin-like staining. The lumen is partially filled by a post-mortem clot. Phosphotungstic acid-hematoxylin stain.  $\times 147$ .

FIG. 18. A longitudinal section of a medium sized coronary artery in the left ventricle of a rabbit sacrificed 3 days after a second large injection of horse serum. The lesion is focal and more severe on one side of the vessel than on the other. The endothelium is well preserved, but the subendothelial tissue is swollen. Medial necrosis (cytolysis) is evident. The adventitia is moderately edematous and contains a few lymphocytes and plasma cells. Hematoxylin and eosin stain.  $\times 266$ .

FIG. 19. A small artery in the right ventricle of the rabbit from which Figures 16 and 17 were prepared. The dark strands in the adventitia are fibrin-like in staining quality; a similar feature also appears in the outer media. There is an inflammatory reaction to perivascular myocardial necrosis. Phosphotungstic acid-hematoxylin stain.  $\times 378$ .

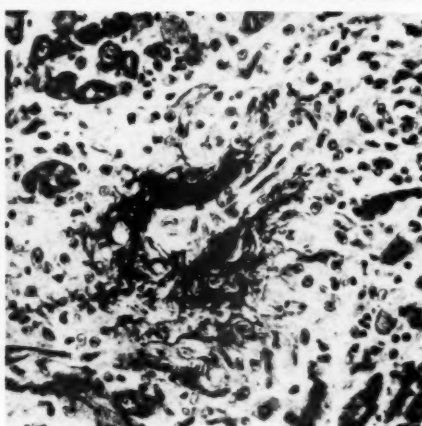
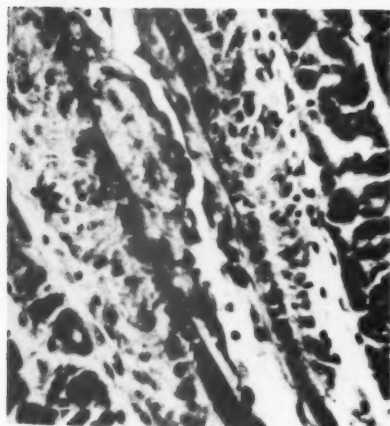
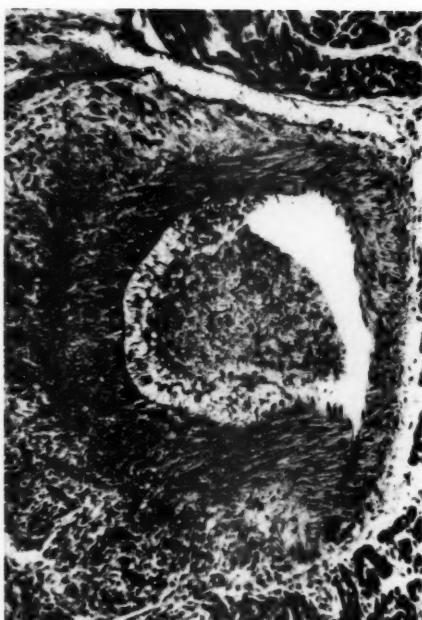
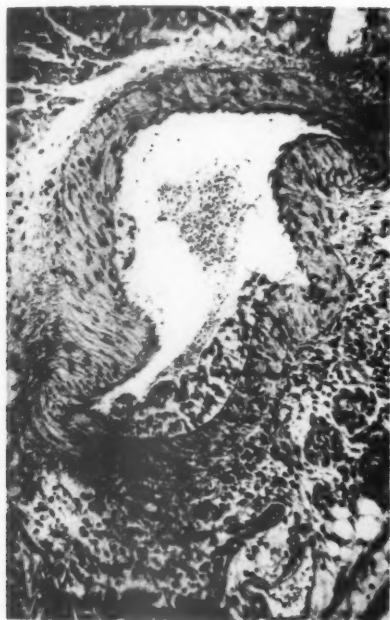
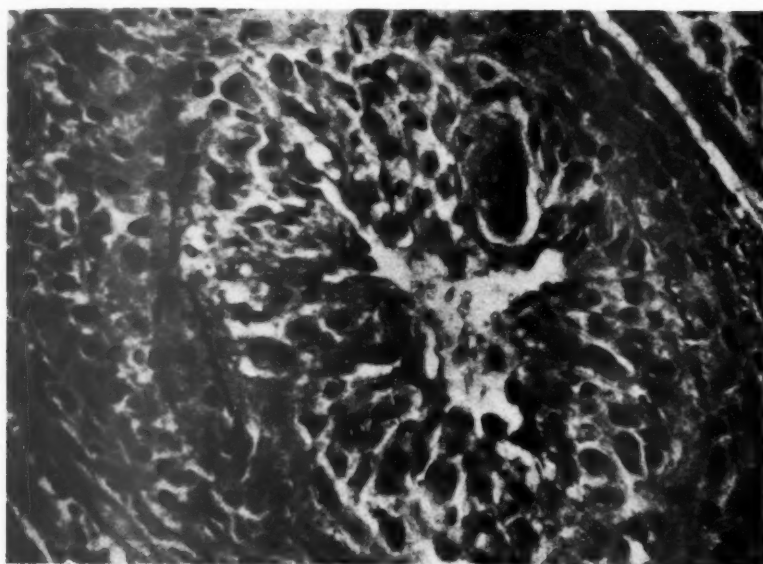


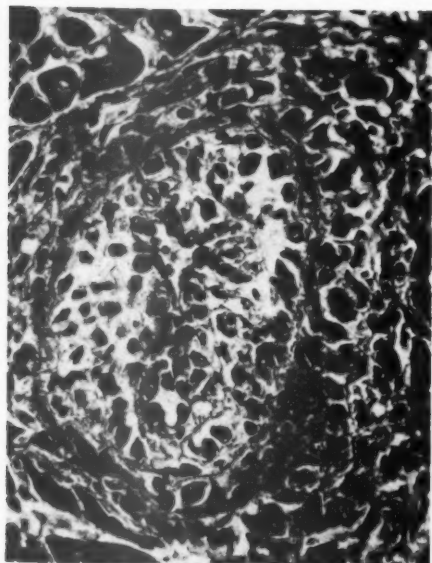
FIG. 20. A large branch of a coronary artery in the left ventricle. Same rabbit from which Figures 16, 17 and 19 were prepared. Note the markedly increased cellularity of the subendothelial, medial and adventitial regions. A multinucleated mass with at least 9 nuclei is present in the upper part of the photograph. No other lesion similar to this was found in the rabbits of this experimental group. The internal elastic lamina is destroyed at many points. Hematoxylin and eosin stain.  $\times 436$ .

FIG. 21. Small artery in the left ventricle of a rabbit sacrificed 9 days after a second large injection of horse serum. This is a healing lesion in which the lumen is narrowed to a slit. There are many cells in the thickened subendothelial region. The media exhibits binucleated cells and disorientation of smooth muscle. Giemsa stain.  $\times 365$ .

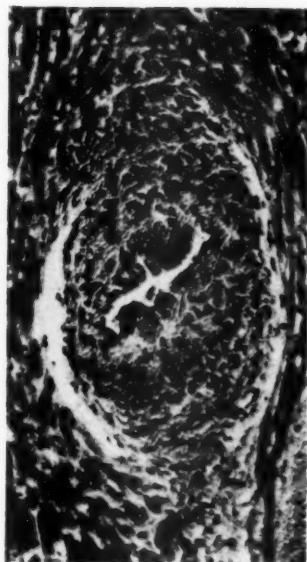
FIG. 22. A medium sized artery in the left ventricle of a rabbit sacrificed 9 days after the second large injection of horse serum. The subsiding inflammatory reaction in the upper and lower portions of the vessel wall indicate healing. There is little or no necrosis or edema. Interlacing fibers of collagen appear as light staining strands extending throughout the wall of the vessel. Thickening of the intima and inflammation in the adventitia are focally distributed. Hematoxylin and eosin stain.  $\times 184$ .



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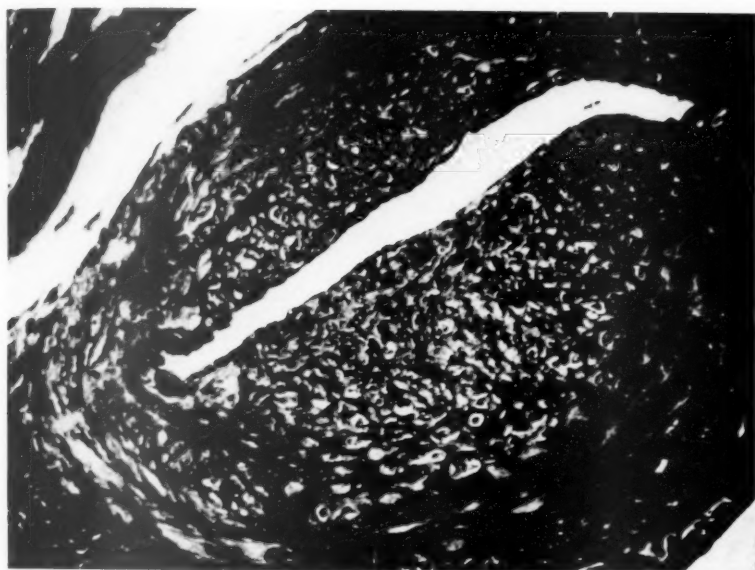


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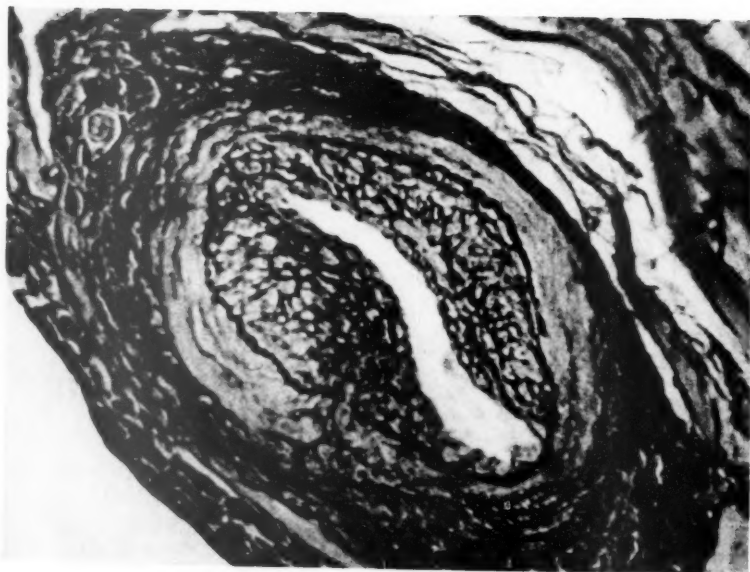
FIG. 23. A medium sized artery in a papillary muscle of the left ventricle of a rabbit sacrificed 31 days after the second large injection of horse serum. A healed lesion with markedly cellular segmental thickening of the intima narrowing the lumen to a slit. Interlacing fibers of collagen are seen in the subendothelial region. Masson's trichrome stain.  $\times 448$ .

FIG. 24. Elastic stain of an artery in the left ventricle of rabbit from which Figure 23 was obtained. In this healed lesion, interlacing bands of collagen extend from the adventitia to the inner layers of the artery. Numerous cells, probably of smooth muscle origin, and interweaving elastic and collagenous fibers thicken the intima and narrow the lumen. However, there is no concentric reduplication of the internal elastic lamina as seen in group I rabbits (Figs. 10 and 12). Weigert-van Gieson stain.  $\times 174$ .

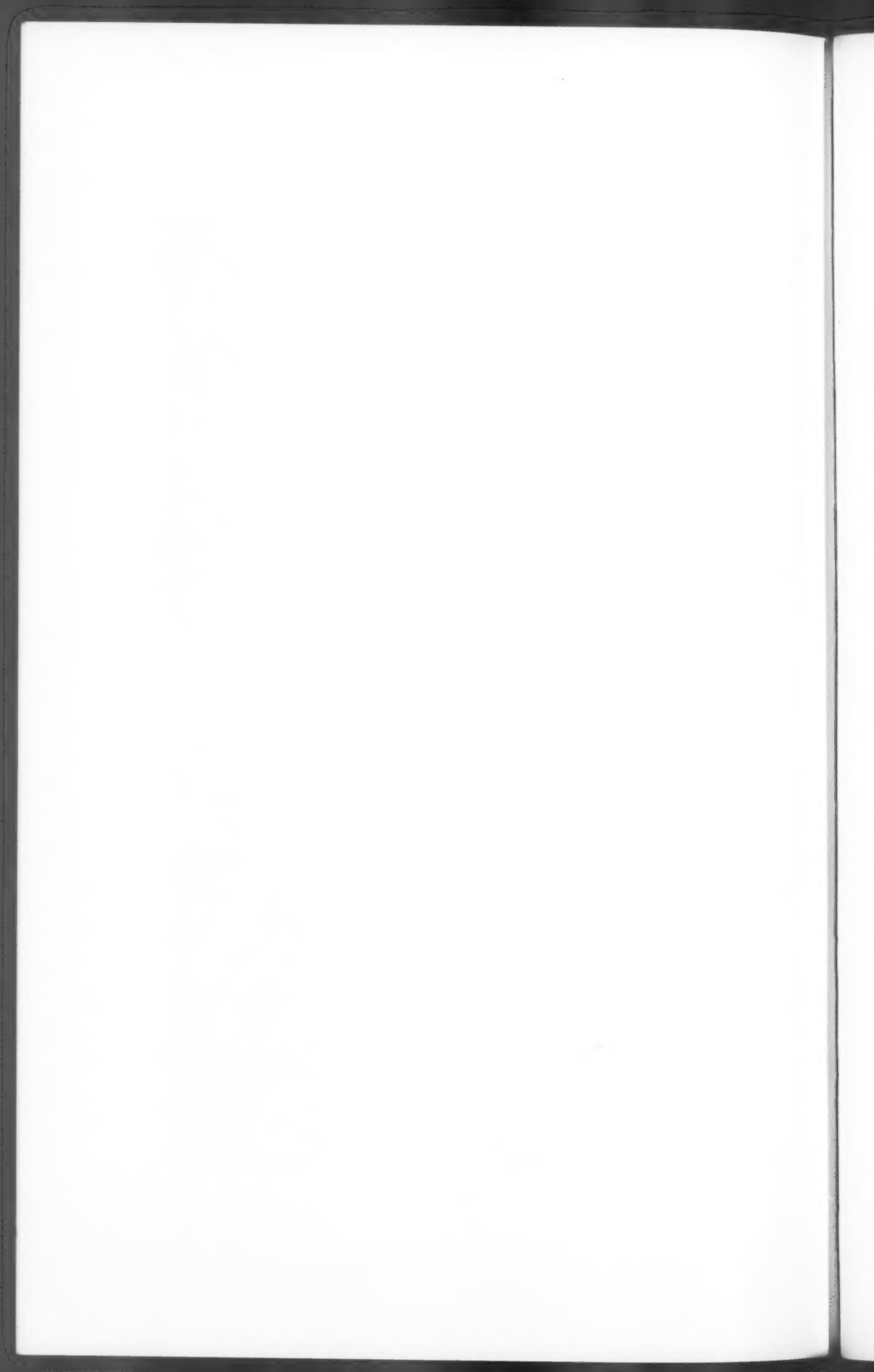




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## THE CARDIAC CONDUCTION TISSUE AND ITS BLOOD SUPPLY IN THE DOG\*

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Probably the first man to tie a coronary artery and see the heart cease to beat afterwards was Chirac in 1698,<sup>1</sup> but his experiment attracted little attention. In fact, interest was only aroused in this problem after John Hunter's death<sup>2</sup> in 1793 in an attack of angina pectoris with the subsequent confirmation of Jenner's prediction<sup>3</sup> that his coronary arteries would be abnormal.

Examination of the atrioventricular node and the bundle of His and its branches in the dog reveals an anatomic distribution which is similar to that of the human being.<sup>4-12</sup> In both species the conduction tissue of the atrioventricular node region and bundle of His is seen to be in direct relationship to a profuse capillary network. As the canine cardiac blood supply is sufficiently similar to the human to make the dog a suitable experimental animal, it is the purpose of this investigation to define the vessels supplying the blood to this capillary bed in the dog, and to investigate the alterations which occur when they are occluded. This has provided us with an opportunity to study infarcts in the interventricular septum and evidence of injury in the conduction tissue.

### ANATOMY

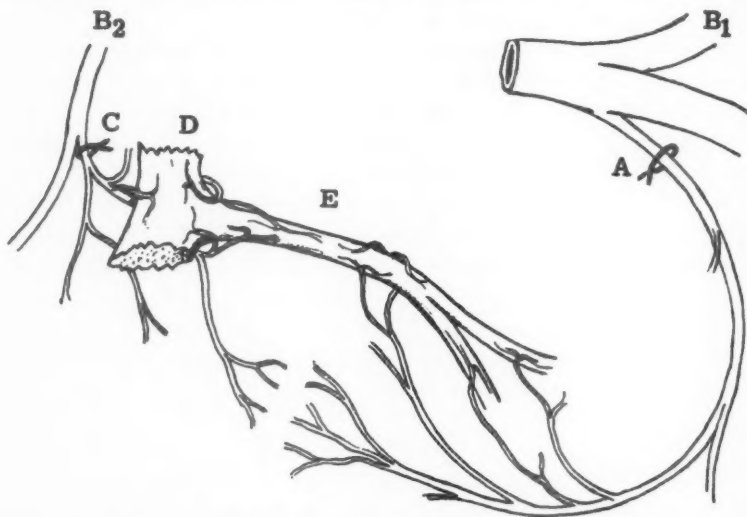
Two principal vessels supply branches to the upper part of the interventricular septum and the floor of the right atrium between the orifice of the coronary sinus and the membranous septum near the root of the aorta (104 dog hearts dissected). One is a posterior vessel which we have termed the "posterior septal artery"; it has also been designated "*ramus septi fibrosi*."<sup>13</sup> The other is an anterior vessel designated the "anterior septal artery." Moore<sup>14</sup> described the anterior septal artery in dogs, naming it the "left septal artery," and pointed out the difficulties of occluding it experimentally in view of its position deep to the left coronary artery.

The posterior artery, in our dissections, arose in all cases from the left circumflex artery near the origin of the posterior descending branch (Text-fig. 1 and Fig. 1). It passed anteriorly just to the left

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and inferior to the termination of the coronary sinus. At this point it lay in fat in a flat plateau formed by the upper surface of the muscular interventricular septum. It continued anteriorly beneath the muscle of the floor of the right atrium, giving branches to the walls of the left and right atrium, and ended in capillaries near the root of the aorta. It passed inferior to the atrioventricular node and followed the course of the bundle of His closely. It was from this vessel that the blood supply to the atrioventricular node and bundle originated. Along its course it gave off arterial twigs which passed



Text-figure 1. Diagram of blood supply to cardiac conduction tissue in the dog.

A, ligature on anterior septal artery

B<sub>1</sub>, origin of left circumflex artery

B<sub>2</sub>, termination of left circumflex artery

C, tie on posterior septal artery

D, atrioventricular node

E, bundle of His

through the *annulus fibrosus* to end in the upper portion of the interventricular septum. A branch to the upper part of the right ventricular wall frequently arose from the posterior septal artery. This anastomosed with the first lateral division of the posterior descending artery. In our experience, on one occasion only was communication demonstrated with terminal branches of the right coronary artery. On 3 occasions, 2 separate vessels arose close to each other in place of the single posterior septal artery.

The anterior septal artery arose from the posterior surface of the left coronary artery at the point of its division or within 2 to 3 mm. of it along the course of the anterior descending artery.<sup>15</sup> It passed directly

posteriorly in the posterior wall of the infundibulum of the right ventricle just inferior to the cusps of the pulmonic valve (Text-fig. 1 and Fig. 2). It entered the interventricular septum and divided to give one branch which proceeded inferiorly, supplying branches to the central part of the upper two thirds of the interventricular septum and moderator band; and another which passed superiorly to the upper part of the septum and ended in a capillary network near the aortic root.

Colored latex injected directly into the anterior septal artery showed dye in the capillary bed of the upper septum as far as the distal portion of the atrioventricular bundle, and dye could also be seen in posterior septal branches, showing evidence of intercommunication between these two vessels.

As a result of these studies, it is our opinion that the blood supply to the major portions of the right and left bundle branches and to the distal one eighth to one fourth of the atrioventricular bundle is from the anterior septal artery. Penetrating muscular branches enter the interventricular septum from the anterior and posterior descending arteries. They probably contribute some blood supply to the more distal parts of the bundle branches, but apart from the uppermost posterior branches, no significant anastomosis seems to occur with the primary supply to the atrioventricular node and bundle of His.

It seems appropriate at this stage to describe briefly the structure of the conduction tissue. We found its anatomic arrangement to coincide with that recorded by others.<sup>18-19</sup> The atrioventricular node lay above the *annulus fibrosus* and below the floor of the right atrium (Fig. 3), near the entrance of the coronary sinus. The bundle extended anteriorly through the *annulus fibrosus* in the lower part of the membranous septum, ensheathed by connective tissue and divided into the right and left bundle branches (Fig. 4) which continued as usually described. The cells of the atrioventricular node formed an irregular, interlacing group closely related to the capillary network already described (Text-fig. 1 and Fig. 5), but in the bundle they were arranged in a more parallel manner. In our specimens the fibers were demarcated from the surrounding structures by a zone of loose connective tissue. The fibers of the bundle branches were larger and showed less well defined cross striations.<sup>20</sup> It is our opinion, based on morphologic appearances, that the conduction tissues are composed of cardiac muscle fibers.

In drawing a comparison with human hearts, it may be stated that essentially similar blood vessels to those described in the dog can be

found. However, the posterior septal artery arises in 92.3 per cent of cases (220 human hearts dissected) from the right coronary artery. The anterior septal artery seems to have a relatively smaller bore in the human subject than in the dog and arises from the posterior surface of the anterior descending branch of the left coronary artery between 15 and 25 mm. from its origin. It seems possible that the uppermost penetrating muscular branches from the anterior descending branch of the left coronary artery provide more anastomosis with the anterior septal artery in the human subject than in the dog, and an attempt to assess the significance of this fact will be a feature of future study. Both anterior and posterior septal arteries pursue identical courses in the two species.

#### EXPERIMENTAL TECHNIQUE

Unselected mongrel dogs of both sexes, varying in weight from 22 to 35 pounds, were used for these experiments. The anesthetic was intravenous 6.5 per cent sodium pentobarbital (1 cc. per 5 pounds of body weight). A left sided approach was made through the fourth intercostal space to expose the anterior vessel. The chest was opened one interspace lower when the heart was to be rotated, in order to display the posterior septal artery. Silk ligatures were used, and complete vessel occlusion was demonstrated in each case (Figs. 1 and 2). Following all the operations, the dogs were given 600,000 units of penicillin intramuscularly. No other medication was given either before or after operation. Standard lead II electrocardiograms were recorded during the operation and at intervals thereafter until the time of death or sacrifice of the animal. In these, only the rhythm patterns have been assessed. After recovery, all dogs were exercised morning and evening and fed regularly once a day. No attempt was made to perform exercise tolerance tests in any of the animals.

*Anterior Septal Artery Dissection.* The avoidance of pressure is required in placing the suture around the vessel, as it is very close to the fragile pulmonary conus. Babcock clamps were used to hold the pulmonary artery, and a spring clip was placed on the left atrial appendage, turning it back from the operative zone. The great cardiac vein may pass very close to the dissection area and require careful retraction. A curved Kelly clamp of small size was used to pass the suture behind the anterior septal artery.

*Posterior Septal Artery Dissection.* Babcock clamps were applied to the fat in the atrioventricular groove, and the heart was pulled around gently until the posterior descending vessel was visualized and the point of entrance of the coronary sinus into the right atrium

TABLE I  
*Experimental Procedures*

| Group | Artery occluded                                 | No. of dogs | Death from operation |      | Size of infarct (gm.) | Infarct % of heart wt. (%) | EKG* evidence of heart block |      |
|-------|---|-------------|----------------------|------|-----------------------|----------------------------|------------------------------|------|
|       |   |             | No.                  | (%)  |                       |                            | No. of dogs                  | (%)  |
| I     | Anterior septal                                 | 11          | 2                    | 18.2 | 6 to 8                | 5.5 to 7                   | 7                            | 63.6 |
| II A  | Posterior septal                                | 20          | 4                    | 20   | 2 to 3                | 2.5 to 3                   | 11                           | 55   |
| II B  | Distal left circumflex                          |             |                      |      |                       |                            |                              |      |
| III A | Anterior septal and complete posterior septal   | 14          | 13                   | 92.9 | Acute experiments     |                            | 11                           | 78.6 |
| III B | Anterior septal and incomplete posterior septal |             |                      |      |                       |                            |                              |      |
| IV    | Anterior descending                             | 6           | 0                    | 0    | 8 to 11               | 8 to 10                    | 4                            | 66.7 |
| V     | Proximal left circumflex                        | 20          | 8                    | 40   | 22 to 36              | 18 to 38                   | 0                            | 0    |
| VI    | Right coronary                                  | 12          | 10                   | 83.3 | 20                    | 17 to 23                   | 3                            | 25   |
|       |   | 10          | 0                    | 0    | 15                    | 12 to 18                   | 0                            | 0    |

\* Electrocardiogram.



was defined. The posterior septal artery was ligated (Fig. 1) and further dissection continued in order to determine whether ancillary vessels arose from the posterior descending artery or there was a double origin to the posterior septal artery. In the event of any of these possibilities, the additional vessels were tied.

When both septal arteries were tied at the same operation, rotation of the heart and posterior septal artery ligation were performed first. The heart was then restored to its normal position and the obliteration of the anterior septal vessel completed.

In addition to these 3 experiments, occluding septal arteries individually and in combination, a control series of dogs was submitted to ligation of various other major coronary arteries.

In this investigation, 104 operations were performed; 11 dogs (10.5 per cent) died as a direct result of operative accidents and were excluded from analysis. The successful experiments in 93 dogs were grouped as follows (Table I):

*Group I. Ligation of the Anterior Septal Artery (11 Dogs)*

One dog died after 28 minutes and one after 18 hours. The other 9 animals were sacrificed at 10 days (1); 14 days (5); 18 days (1); 21 days (1); and 56 days (1). The heart of the dog which died after 18 hours contained a thrombus extending from the orifice of the anterior septal artery and partly occluding the lumen of the anterior descending branch of the left coronary artery. Extension of the clot was not observed in any of the other dogs in this group and may have contributed to the early death in this case.

The dogs which survived slept a great deal during the first 48 hours after operation but, with two exceptions, fed normally and recovered completely thereafter. In the two exceptions episodes of dyspnea, melena and hematuria developed; these were considered to be manifestations of cardiac failure.

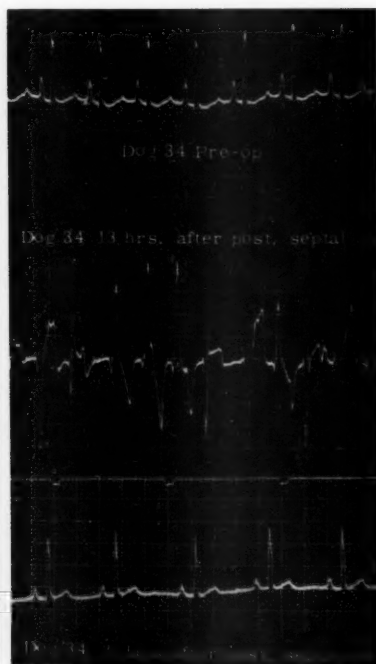
Seven of the dogs exhibited abnormalities of conduction. In two this was characterized by a prolongation of the PR interval which appeared 24 to 56 hours after operation and lasted for 12 and 24 hours respectively. In 3 the electrocardiogram exhibited heart block varying from 3 to 1 to 6 to 1. In one animal the block persisted until sacrifice on the 56th day (Text-fig. 2). The other two had grade II block, which persisted for 3 days after operation, after which normal rhythm was restored.

On dissection, infarcts were found which were confined to the upper two thirds of the interventricular septum, extending to the endocar-

dium of the left side but sparing 2 to 5 mm. of subendocardial region on the right side in the lower three quarters (Fig. 7). The weight of the infarcts varied between 6 and 8 gm. and constituted 5.5 to 7 per cent of the total heart weight. Microscopic examination showed typical healing infarcts with destruction of the septal muscle and evidence



Text-figure 2. Electrocardiogram showing complete heart block following anterior septal artery ligation.



Text-figure 3. Electrocardiogram showing grade II heart block following occlusion of posterior septal artery.

of some destruction of the distal part of the bundle of His and bundle branches. A constant feature was the maintenance of viable fiber groups of the conduction tissue in the regions in which all other tissue was necrotic (Figs. 8 and 9).

#### *Group II A. Ligation of the Posterior Septal Artery (11 Dogs)*

Nine of the dogs survived more than 24 hours and were sacrificed at 8 days (2); 14 days (6); and 26 days (1). The two dogs which died developed ventricular fibrillation quite suddenly, 5 minutes and 15 minutes after the vessel had been ligated. The surviving dogs recovered uneventfully from anesthesia, fed well and were exercised regularly. Four animals exhibited grade II heart block for periods

of 5 to 12 hours, 24 to 48 hours after operation (Text-fig. 3). There were no examples of complete heart block. After 72 hours electrocardiographic tracings showed only some slurring of the ST segment.

In 3 dogs additional blood supply from the first branch of the posterior descending artery was encountered at operation. At necropsy all hearts contained infarcts confined to the upper and posterior portion of the interventricular septum, affecting approximately one fifth of the septal mass (Fig. 10). The weights of the infarcts were approximately 2 to 3 gm. and represented 2.5 to 3 per cent of the total heart weight. No abnormalities were found elsewhere in the hearts. Microscopic examination showed the lesions to be typical healing infarcts with evidence of injury to the atrioventricular node and bundle of His (Fig. 11). In all cases, however, variable numbers of viable fibers remained in the lesions (Fig. 12).

It seemed likely that blood supply from the anterior septal artery and from vessels communicating with the posterior septal vessel provided enough blood supply to maintain adequate conduction.

*Group II B. Ligation of the Distal Left Circumflex Artery  
(9 Dogs)*

The technique of tying this artery immediately proximal to the posterior descending artery and the origin of the posterior septal artery was essentially the same as that used in tying the posterior septal artery. Two dogs died 12 and 20 hours postoperatively, and the remaining 7 survived and were sacrificed at 14 days. Electrocardiographic evidence of atrioventricular dissociation was demonstrated in 7 of the 9 dogs. In two there was complete heart block. At necropsy the infarct produced was found to affect an area of the septum identical to that observed in group II A (Fig. 10). In addition, there was also infarction of a small portion of the upper posterior wall of the right ventricle at its junction with the interventricular septum. Microscopic alterations were similar to those observed in group II A.

*Group III. Ligation of the Anterior and Posterior  
Septal Arteries (20 Dogs)*

Before an assessment of the survival from this procedure is made, the multiple vessel supply to the posterior septal region must be considered. In group II A (posterior septal artery ligation) 3 of the 11 dogs (27.2 per cent) examined showed more than one vessel in this

location. In group III, this phenomenon was demonstrated in 6 of the 20 dogs (30 per cent). For this reason, group III was dealt with as follows:

*Group III A.* This comprised 14 dogs in which there was no gross evidence of vessels to the posterior septal region except those ligated. Thirteen of these died following ligation of both septal arteries at the same operation (92.9 per cent). Eight survived for 20 to 35 minutes; one each survived for 1, 2, 3 and 7 hours. In 3 of these dogs, 2 ligatures were applied in the posterior area. One dog died after 56 hours, and one survived to be sacrificed at 14 days.

*Group III B.* Six dogs had arteries supplying the posterior septal region in addition to those ligated. All these survived the dual vessel ligation operation; half of these were sacrificed at 5 days and half at 14 days.

The 7 dogs which survived the ligation procedure (1 in group III A and 6 in group III B) were somnolent for 48 hours after operation and then slowly attained normal activity after 5 days. Five of these had electrocardiographic evidence of complete atrioventricular dissociation. Three had persistent heart block for 4 days and a fourth for 3 days. After this time, normal rhythm was restored, and only myocardial damage patterns were seen. Ten of the 13 dogs which died after ligation had complete dissociation of rhythm, and death followed progressively increasing heart block so that the mechanism is best described as cardiac arrest. The other 3 dogs developed sudden ventricular fibrillation.

No significant morphologic alterations were observed in the hearts of the dogs which died up to 7 hours after operation. The dog succumbing after 56 hours showed acute myocardial infarction in the upper two thirds of the septum and microscopic evidence of early coagulation necrosis in the region of the atrioventricular node and bundle of His (Fig. 6). The hearts of the surviving dogs contained infarcts affecting the upper two thirds of the septum. The upper and posterior portion, the area supplied by the posterior septal artery, was often spared. The weights of the infarcts ranged from 8 to 11 gm. and comprised 8 to 10 per cent of the total heart weight. The microscopic appearance of the lesions resembled that observed in group I (anterior septal artery ligation). There was considerable damage to the septal myocardium and some injury to the conduction tissue, but in each instance surviving, apparently viable fibers remained.

*Group IV. Ligation of the Anterior Descending Branch  
of the Left Coronary Artery (20 Dogs)*

Twelve dogs survived this procedure and were sacrificed after 14 days. Eight dogs died as a result of the operation; 6 succumbed 25 to 40 minutes after the ligature was applied; one died after 15 hours and one after 3 days.

In all instances, ventricular extrasystoles were noted 20 to 25 minutes after ligation. In the dogs which died at this stage, ventricular fibrillation supervened. No dog in this group showed evidence of rhythm dissociation. Those which survived recovered from the anesthetic quickly and fed and moved about normally. The surviving animals had infarction of the entire thickness of the anterior wall of the left ventricle distal to the ligature. The site of ligation varied from 6 to 18 mm. beyond the point of origin of the artery. It was distal to the anterior septal artery and proximal to any major division of the anterior descending artery. The size of the infarcts bore no constant relationship to the weight of the heart and was presumably dependent upon the effectiveness of collateral circulation. The infarcts weighed from 22 to 36 gm., constituting 18 to 30 per cent of the total heart weight.

*Group V. Ligation of the Proximal Portion of the  
Left Circumflex Artery (12 Dogs)*

Two dogs in this group survived more than 24 hours. One of these died after 2½ days; the other survived and was sacrificed after 30 days. Five animals died within 30 minutes of ligation; 3 survived 10 to 12 hours, and 2 lived 15 hours. Three dogs had grade II heart block (those surviving 15 hours, 2½ days, and 30 days). In 8 instances the tie lay 1.5 to 2 cm. from the origin of the vessel; in 4 dogs it lay 4 to 8 mm. from the point of origin. Twice the ligature was found to be proximal to a left atrial branch, but it was proximal to the main lateral marginal vessel in all instances. The ligatures in the dogs surviving 2½ and 30 days lay 2 cm. from the origin of the artery, and no features distinguished these hearts from those in dogs which died within 30 minutes when the tie was at the same point. Myocardial infarcts were seen only in the 4 dogs in this group which survived for 15 hours or longer. In these, the anterolateral and posterior wall of the left ventricle and a small area of the posterior and upper portion of the interventricular septum were affected. This was similar to but smaller than the area of infarction induced when the

distal left circumflex or posterior septal artery was tied (group II). This fact, in conjunction with the lower incidence of dissociation of conduction in this group, seems to indicate that better collateral circulation is available in the terminal drainage area to the left circumflex artery when the occlusion is near its origin. The 4 infarcts in the cases which survived for 15 hours or longer averaged 20 gm. Microscopically, the typical alterations of fresh and healing infarcts were observed. In the septal region, evidence of injury to the atrioventricular node and the proximal portion of the bundle of His was demonstrated. Here, also, however, there were many fibers spared and apparently in normal state.

#### *Group VI. Ligation of the Right Coronary Artery (10 Dogs)*

In this group, operative approach was carried out through the fourth right intercostal space. The procedure was attended by no mortality. All animals recovered from the anesthetic quickly, fed and exercised well and were sacrificed on the 14th postoperative day. Electrocardiographic tracings showed well marked waves indicative of trauma but no disturbance of rhythm. The site of ligation varied from 1 to 1.3 cm. from the origin of the vessel. In all cases one atrial branch vessel arose proximal to the tie. Myocardial infarcts involving the lateral and posterior wall of the right ventricle and part of the posterior wall of the right atrium were present in all cases. Their weight varied only slightly from an average of 15 gm., which constituted 12 to 18 per cent of the total heart weight. Microscopically, the lesions had the characteristics of healing infarcts. The interventricular septum was unaffected in all instances.

#### DISCUSSION

In 1893 Porter<sup>21</sup> reported two instances in which, following the experimental ligation of the anterior septal artery (*ramus septi*), the heart's action was observed for 30 minutes without death occurring. He described the difficulty of the procedure utilized; it was probably the result of this technical problem that most subsequent experiments were confined to ligation of the larger superficial arteries, accessible without extensive manipulation of the heart. Our investigations have shown that ligation of the anterior and posterior septal arteries results in infarction of the interventricular septum and to a variable extent, the atrioventricular node, bundle of His, and origin of the bundle branches. The mortality following ligation of each vessel separately was low, but electrocardiographic evidence of damage to the conduc-



tion tissue was found in more than half the cases (group I, 63.6 per cent; group II, 55 per cent). Microscopically detectable infarction was regularly induced although uninjured myocardial fibers were found in each infarct. Our experiments indicate that anastomosis exists between the anterior and posterior coronary arterial supply, sufficient in the majority of animals to maintain viability of a significant number of conduction fibers.

The very high mortality which resulted from occlusion of both vessels at the same operation would indicate that there is no other significant blood supply to the conduction system. Experiments for the purpose of inducing myocardial muscle injury in other regions of the heart have been effected by occluding other coronary arteries and their branches. The mortality rates recorded for these arterial occlusion experiments vary considerably. For anterior descending artery ligation, a mortality rate of approximately 10 per cent was given by McEachern, Manning and Hall<sup>22</sup> and Chardack and associates.<sup>23</sup> On the other hand, Carter, Gall and Wadsworth<sup>24</sup> cited the rate of 48 per cent; Bakst, Costas-Durieux, Goldberg and Bailey,<sup>25,26</sup> 60 per cent; Beck and his colleagues,<sup>27,28</sup> 70 per cent; and Vineberg,<sup>29,30</sup> 80 to 90 per cent. Allen and Laadt<sup>31</sup> emphasized the importance of stating the location of ligation of the vessel accurately and indicating the number of branches proximal to the ligature site. They showed that following ligation of the left circumflex artery, the mortality rate varied inversely with the proximity of the ligature to the origin of the vessel.

The myocardial infarcts encountered in groups IV, V and VI were considerably larger than those observed following anterior and posterior septal artery occlusion (Table I). This suggests specialized function in the upper interventricular septum. Corroborative evidence for this was supplied by the high frequency with which electrocardiographic evidence of dissociation of rhythm was encountered. Although the evidence obtained in our experiments cannot be regarded as conclusive, it seems reasonable to assume that injury to conduction tissue fibers in the upper interventricular septum is the factor which makes ischemia of this region such a lethal condition. It is tempting to entertain the possibility that the higher mortality recorded following left circumflex artery ligation in this and other studies, as compared with that following occlusion of the anterior descending artery distal to the anterior septal branch, could be attributed to the involvement of conduction tissue when left circumflex arterial flow is interrupted. Wood and Wolferth<sup>32</sup> suggested that the circumflex branch supplied



a region of the heart peculiarly prone to ventricular fibrillation when rendered ischemic.

In attempting to correlate the experimental observations with human disorders, two major points of interest emerge. The first is concerned with the possibility of conduction tissue injury in the course of surgical procedures in the region of the interventricular or interatrial septums. The second point relates to the significance of vascular obstruction when either of the vessels discussed above is partially or completely occluded as the result of intrinsic abnormality.

#### SUMMARY

Ligation of arteries supplying the atrioventricular node and bundle of His and its branches has been carried out in the dog. The myocardial alterations induced by occlusion of either the anterior or posterior septal arteries or both are described. A series of "control" experiments have been performed in which other coronary arteries and their branches were ligated. The significance of ischemia in the region of the upper interventricular septum in the dog is discussed and the importance of correlating these observations with human myocardial lesions is emphasized.

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[ Illustrations follow ]

## LEGENDS FOR FIGURES

- FIG. 1. Origin of posterior septal artery, showing occluding ligature. The coronary sinus is seen above and the posterior descending artery below.
- FIG. 2. Origin and distribution of the anterior septal artery. Black silk can be seen occluding its point of origin.
- FIG. 3. Normal AV node and commencement of the bundle of His above and to the right of the *annulus fibrosus*. Hematoxylin and eosin stain.  $\times 22.5$ .
- FIG. 4. Normal AV bundle showing origin of right and left bundle branches. Hematoxylin and eosin stain.  $\times 16$ .



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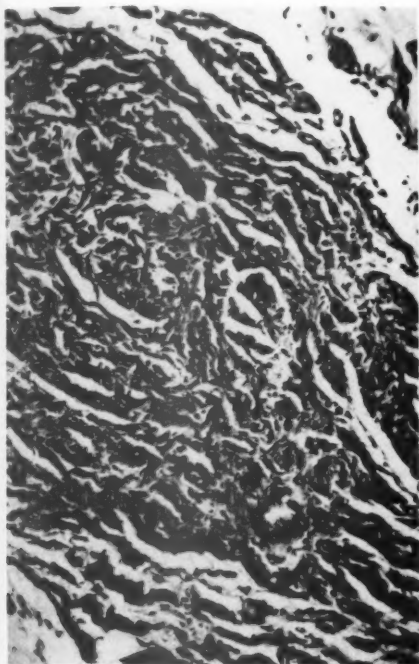
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FIG. 5. Normal AV node. Hematoxylin and eosin stain.  $\times 65$ .

FIG. 6. Recent infarct of conduction tissue following ligation of both the anterior and posterior septal arteries. Dog died 56 hours after operation. Hematoxylin and eosin stain.  $\times 65$ .

FIG. 7. Interventricular septum viewed posteriorly, showing infarct resulting from anterior septal ligation. Dog sacrificed after 14 days.

FIG. 8. Infarct resulting from anterior septal artery occlusion in relation to left bundle branch. Dog sacrificed after 14 days. Hematoxylin and eosin stain.  $\times 120$ .



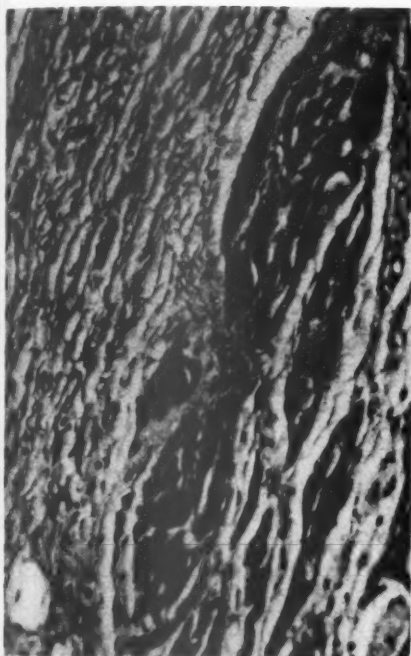
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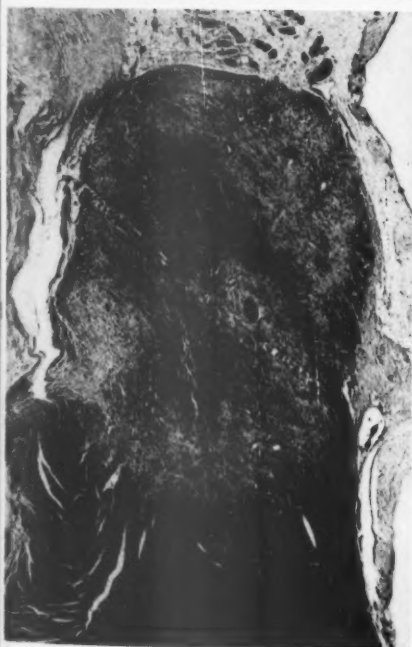
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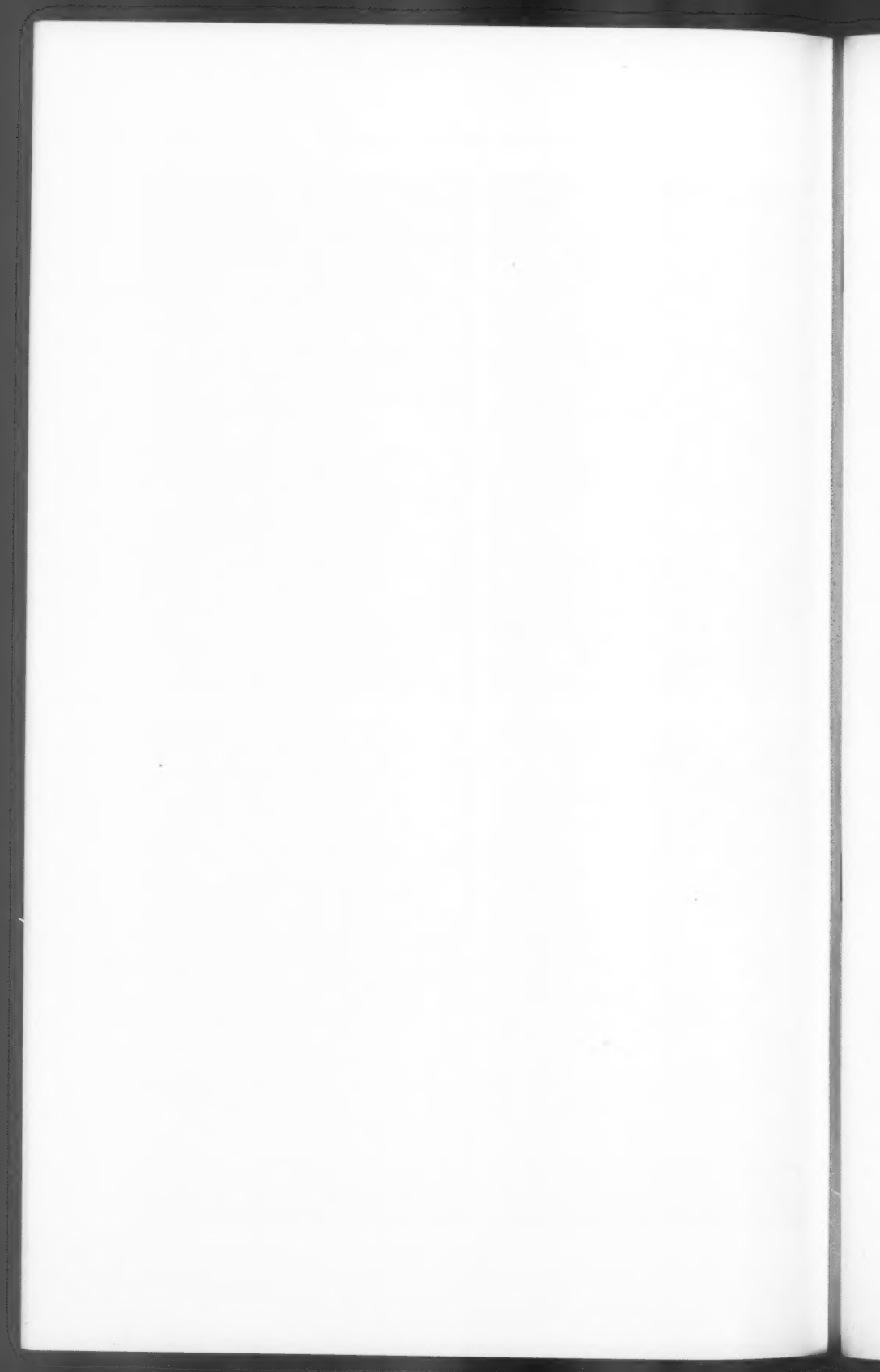
- FIG. 9. Fibrosis and surviving AV bundle fibers following anterior septal artery ligation. Dog sacrificed after 14 days. Hematoxylin and eosin stain.  $\times 120$ .
- FIG. 10. Interventricular septum viewed posteriorly, showing area of infarction resulting from posterior artery ligation. Dog sacrificed after 14 days.
- FIG. 11. Infarct of upper part of interventricular septum, largely destroying conduction tissue following posterior septal artery ligation. Dog sacrificed after 14 days. Hematoxylin and eosin stain.  $\times 30$ .
- FIG. 12. Group of surviving conduction fibers in area of infarction following posterior septal artery ligation. Dog sacrificed after 14 days. Hematoxylin and eosin stain.  $\times 60$ .



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## MYOCARDIAL ISCHEMIA AND EARLY INFARCTION: AN ELECTRON MICROSCOPIC STUDY\*

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This investigation is part of a study of the early morphologic alterations in myocardial ischemia and infarction produced by ligation of the coronary artery of the rabbit. Tissue obtained from the experimental animals was investigated by various histochemical techniques and by electron microscopy. Ventricular myocardium was selected because the cells here are relatively uniform in structure and function. It was hoped that by varying the period of time between arterial ligation and fixation of the tissue, the sequence of cellular events could be elucidated. This report deals primarily with the electron microscopic features.

Experimental ligation of the coronary arteries has been performed in a wide variety of animals. Although chemical determinations have revealed losses of glycogen and increases of lactic acid within a few minutes after coronary occlusion,<sup>1-3</sup> the effects of ischemia were not usually noted histologically before 4 to 6 hours had elapsed.<sup>4,5</sup> Chemical measurements have revealed marked loss of glycogen in anoxic hearts within 4 minutes.<sup>6</sup> Progressive chemical and histologic alterations of infarction did not follow temporary occlusions of less than 20 to 30 minutes.<sup>7</sup> The earliest histochemical change recorded is the loss of glycogen in from 30 to 60 minutes.<sup>7,8</sup> In view of the evidence indicating loss of glycogen within minutes and the apparent induction of necrobiosis within 30 minutes, it was felt that structural alterations not observable by the light microscope might be demonstrated with the electron microscope.

### MATERIAL AND METHODS

Male and female albino rabbits weighing 4 to 5 pounds were anesthetized with intravenous sodium pentobarbital (50 to 75 mg.). Following tracheotomy and intubation, a left lateral thoracotomy was performed, using positive pressure respiration. After opening the

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pericardium, a small curved-eye needle was passed deep to a branch of the left coronary artery, which was occluded by ligation with a cotton thread. Within one minute after successful arterial occlusion, the affected myocardium blanched and subsequently became cyanotic. The lung was then re-expanded, and an airtight closure of the chest in multiple layers was performed.

After a chosen interval, the chest was reopened through the previous incision, and the entire heart was removed and transected below the ligature. This second procedure was carried out with positive pressure respiration and additional anesthesia as required. Eighty rabbits were operated upon and investigated by histochemical methods. Electron microscopic studies were carried out in 37 of the experimental hearts and in 6 control hearts. In the experimental animals the intervals were from 5 minutes to 5 hours after coronary ligation. Animals were excluded from the study if a vein was inadvertently included in the ligature or if the characteristic blanching and cyanosis did not occur.

A slice of the ischemic region was removed, cut into cubes measuring 1 to 2 mm. on a side, fixed in buffered osmium tetroxide, dehydrated in alcohols, and embedded in methacrylate.<sup>9-11</sup> Numerous muscle blocks from non-ischemic portions of damaged hearts were processed in similar fashion. Thin sections of the methacrylate blocks were cut with a Porter-Blum microtome and mounted on grids with carbon films.<sup>11-14</sup> All photographs were taken with an RCA EMU 3B at initial magnifications of 3,000 to 12,000. Adjacent blocks of heart tissue were processed for histochemical studies. These will be reported subsequently.

## RESULTS

### *Normal Hearts*

The structure of normal cardiac and skeletal muscle has been well described.<sup>15-21</sup> Although there has been disagreement concerning the surface structure,<sup>22,23</sup> the anatomic features relevant to the present investigation are depicted in Figures 1 to 3. In electron micrographs the surface membrane was clearly demonstrated to be a complex membrane system consisting of two dense areas separated by a lighter zone<sup>15,17</sup> (Figs. 1 to 3). The membrane complex is referred to as the sarcolemma which is adherent to the myofibrils at the "Z" band. As can be seen in Figure 2, this indented and was continuous with the endoplasmic reticulum, a structure which is well developed at the "Z" bands.<sup>16,17</sup> Small vesicles in the sarcoplasm were manifest near the membrane. In sections stained with the periodic acid-Schiff (PAS) stain, the sarcolemma was magenta and continuous with the intercalary discs. The PAS-positive material was not removed by diastase. The

general distribution and continuation with intercalary discs indicated that the sarcolemma (Figs. 1 and 3) was identical with the PAS-positive membrane observed with the light microscope. Specimens prepared by freeze drying or freeze substitution and stained with PAS demonstrated the polar localization of magenta-staining, perinuclear material which was removed by diastase. Figure 3, an electron micrograph from a region with abundant glycogen, demonstrates many granules 150 to 250 Å in diameter which were not sharply defined and were not associated with the endoplasmic reticulum as in the case of ribonucleic acid (RNA) granules.<sup>24</sup> The former have been tentatively identified as glycogen.<sup>20,21</sup> Although glycogen, as demonstrated by PAS staining and diastase, appeared to be located in other regions of the sarcoplasm, in electron micrographs it was clearly seen only around the nucleus.

The bundles of myofilaments, which occasionally branched, were frequently surrounded by mitochondria and elements of the endoplasmic reticulum (Fig. 1). Usually a mitochondrion was found in the fork of a branch. Cross sections of myofibrils showed close packing of filaments with hexagonal configuration (Fig. 8).<sup>19</sup>

In sections of fixed normal heart, contraction bands were generally in the region of the "Z" band (Fig. 1). These were the result of placing pieces of muscle directly in fixative with no attempt to stretch or maintain fiber length. In most longitudinal sections, a dense "H" zone appeared halfway between two "Z" bands. "I" bands were not visible in any of the normal, contracted preparations; however, "A" bands were prominent. Myofilaments coursed through a given cell and terminated at intercalary discs.<sup>20,21</sup>

Mitochondria were found throughout the heart muscle fiber. They were located about the nucleus at either pole, often in the same regions where glycogen was demonstrable. They were also found in long chains between bundles of myofilaments (Figs. 1 and 3). The structure of these bodies was similar regardless of their location within the myocardium.<sup>25</sup> The mitochondrial membranes were the sites of the greatest concentrations of the oxidative electron transfer systems.<sup>26,27</sup> The endoplasmic reticulum of striated muscle consists of tubules, vesicles and cisternae.<sup>16-18</sup> These are organized about the myofibrils in units that repeat with the sarcomeres. The endoplasmic reticulum of cardiac muscle was similar to that of skeletal muscle (Figs. 1 to 3). Its association with the sarcolemma is shown by the arrow in Figure 2. Points of continuity between the endoplasmic reticulum and the external portion of the nuclear envelope, described in other cell types, have been seen in heart muscle also.<sup>28</sup>

The nucleus was bounded by a double membrane (Fig. 3). Nucleoplasm was evenly distributed, without dense aggregates. The nuclear material itself was comprised of a random array of filamentous structures and denser granules. Nucleolar substance frequently appeared as an aggregate of closely packed granules.

### *Myocardial Infarction*

Morphologic alterations occurred quickly in ischemic muscle. Five minutes after ligation of a coronary artery, perinuclear PAS staining was markedly decreased in intensity. This was reflected in electron micrographs by the absence of perinuclear glycogen particles of the order of 150 to 250 Å (Fig. 5). At no time after the onset of ischemia were these particles evident. There was a sharp division between fibrils having a normal glycogen content and those in which it was absent. The regions previously occupied by glycogen were now pale, and the endoplasmic reticulum was more prominent. There was no position shift of the mitochondria, endoplasmic reticulum, nucleus, or myofibrils in the regions depleted of glycogen. The fine fibrillar background was undisturbed by the loss of glycogen.

Light microscopy clearly demonstrated the sarcolemma in regions rendered ischemic for 12 hours. Electron micrographs of areas ischemic for 5 hours revealed rupture of sarcolemma but no disturbance of the spatial relationships of the dense and pale regions (Fig. 12). The external membrane was still in continuity with collagen fibrils. The middle pale layer was not swollen, nor was there any evidence of fibrillar material. The dimension of the inner membrane was still 80 Å; however, there was a decrease in number of the small vesicles formerly seen touching it and adjacent to it. This decrease was gradual and was first apparent about 1½ hours after arterial ligation. There was evidence of slight separation of adjacent cells in preparations ischemic for 10 to 15 minutes. In view of cellular shrinkage during fixation and dehydration and the minimal increase in intercellular space, this early change could represent artifact.

A definite accentuation of the intercalary discs was evident in the specimens ischemic for one hour or more (Fig. 10). Adjacent to the disc, an intracellular pale zone appeared which contained a few randomly placed fine filamentous structures. The vacuole formation associated with decrease in density of the entire muscle cell accentuated the darker intercalary disc. The distance between two cells at an intercalary disc did not change appreciably during 5 hours.

Within 5 minutes of arterial ligation, intracellular spaces appeared between myofilaments in the perinuclear region and around the



mitochondria (Fig. 4). Later these spaces increased in size and number. For one-half hour these spaces contained a loose array of very fine filamentous structures; subsequently they appeared empty. At half an hour the bundles of myofilaments showed longitudinal cracking and separation. The separation of myofilaments was random, since the size of the resulting bundles was variable. After 4 to 5 hours, transverse tearing of myofilaments was evident.

Comparison of tissues anoxic for 20 and 35 minutes revealed a marked change in the mitochondria. At 20 minutes they were almost normal, whereas at 35 minutes they had begun to swell. The cristae appeared more distinct because of decrease in the density of the matrix and separation. Throughout this period of swelling, the cristae maintained a rough semblance of their previous order in that they all projected perpendicularly from the surface toward the center. Because of swelling they did not approach the opposite side as closely. After 4 hours of ischemia the cristae of many mitochondria had disappeared while the cristae of others had lost spatial organization (Fig. 11). In a number of instances the limiting membrane was altered, and the overall appearance was similar to that of ruptured mitochondria.<sup>26</sup> The size of the ruptured mitochondria was decreased in relation to the swollen ones observed at 2 to 3 hours. In the specimens procured after 5 hours or longer, collapsed mitochondria were frequent but swollen ones still remained.

During the periods of cellular swelling the endoplasmic reticulum underwent similar modification. The first definite evidence of alteration of the tubules was seen in the specimens at 35 minutes in that simple enlargement occurred. They contained no electron-dense material. At 4 and 5 hours the swelling was more pronounced. Again because of the separation of myofibrils and surrounding loss of density, in the later preparations the system was more clearly seen. Similar alterations, i.e., swelling and enlargement of the vesicles and tubules, also could be seen in the Golgi apparatus near nuclei.

A unique feature of muscle tissue is the presence of various bands under different conditions of stretch. In our investigation two states of contraction were clearly visible. In most of the tissue ischemic for periods up to 20 minutes, the muscle showed contraction bands at the "Z" region, and darkening of the sarcomere midway between the contraction bands was often apparent. In muscle ischemic for 30 minutes or more, contraction bands were absent, but "Z," "I," "A," "H," and "M" bands were evident in most longitudinally sectioned fibers.

The alterations induced by ligation of rabbit coronary artery have

been well described.<sup>29</sup> Two features were very clearly demonstrated by electron microscopy: preservation of the subendocardial tissue, and extremely sharp limits to the lesion. Repeated examinations at the margins of lesions indicated that the transition zone between normal tissue and that maximally damaged measured about 0.1 mm. Within the area of ischemia the extent of damage was variable, so that adjacent fibers showed different degrees of swelling, vacuolization, nuclear alteration and the other features described above. Sections from muscle anoxic for 22 hours frequently exhibited cells undergoing dissolution and immediately adjacent cells still apparently capable of contraction.

Nuclear ischemic alterations were detectable within 5 minutes (Fig. 5). Early, the nucleoplasm showed clumping, and adjacent areas were the seat of rarefaction. Aggregation of nucleoplasm at the nuclear envelope was accentuated with prolongation of ischemia.

The degree of damage manifest in the muscle from one heart after 20 minutes of ischemia was comparable to that seen in the 3 and 4 hour preparations (Fig. 8). It was noted at the time the heart was removed from the carcass that it was fibrillating. The same reaction pattern was observed in two other hearts fibrillating at the time of removal, and it was assumed that the marked damage was attributable to fibrillation.

In order to determine whether the alterations were a result of curtailed blood flow, small fragments of normal heart tissue were excised and placed in .01 M Krebs-Ringer phosphate buffer with sufficient sucrose added to provide an osmolarity in the neighborhood of .34 (assuming 100 per cent ionization of the electrolytes). The tissue was incubated for 3 hours at 37° C. As shown in Figure 14, there was a deviation from normal, but the overall change was slight and not comparable to that seen 3 hours after coronary ligation.

The myocardium contains a number of other tissues than muscle. The fibrous tissue exhibited no change in the abundant material evaluated during periods up to 5 hours after ligation. The vascular endothelial cells developed cytoplasmic and nuclear alterations, though more slowly, than those noted in muscle.

Not uncommonly, red cells seemed to lose hemoglobin (Fig. 12). Since such a small portion of any cell was present in an electron micrograph, the overall volume could not be determined; however, the red cell margins were not crenated although the cells appeared to be swollen. In a number of instances red cells exhibiting leaching lay adjacent to red cells showing no loss of hemoglobin. Apparently, 2 to 3 hours after ligation there was an extensive diapedesis of both

red cells and neutrophils. At no time could a break in capillary continuity be detected, nor could a cell be seen in the process of leaving a vessel.

#### DISCUSSION

A reasonable prelude to any discussion of observations should include consideration of possible sources of error. In these experiments many such sources existed, and some are not well understood. Procurement of specimens required two thoracotomy procedures with positive pressure respiration and on occasion two administrations of sodium pentobarbital anesthesia. It has been shown that 5 to 10 minutes of breathing 10 per cent oxygen is sufficient stimulus to reduce the cardiac glycogen by 80 per cent or more.<sup>6</sup> The general structure and the glycogen content were similar in both the control myocardiums and in the non-ischemic regions of hearts 3 hours after coronary ligation. It would seem reasonable, therefore, to discount the effects of the surgical and related trauma.

Alterations due to fixation are difficult to evaluate in electron microscopy since the appraisals of artifacts of this nature have been gathered from preparation techniques for conventional microscopy. Anoxia may alter tissue response to osmium tetroxide and other components of the fixative. This might be a constant artifact, or it might easily fluctuate in kind as well as degree under anoxic conditions. To our knowledge there is no satisfactory method of evaluating this point.

It has been demonstrated by Ring<sup>20</sup> that the extent of infarction induced by experimental coronary occlusion cannot be determined until approximately 5 days after ligation. The degree of collateral circulation in normal heart muscle has not been established.<sup>30</sup> The existence of a collateral circulation could result in inconstant damage to muscle fibers thus introducing another variable factor.

Our electron microscopic investigations have shown that very shortly after the blood supply to the myocardium was interrupted, clear spaces appeared in various portions of the muscle. It was apparent that these represented an acquired alteration since they were not present in the normal state and they occupied positions in the cell previously held by normal components. Early, the spaces had a fibrillar background similar to that in the normal cell matrix but less dense. Within 30 minutes the matrix became sufficiently diluted to become invisible.

The easiest explanation of this phenomenon is to assume an influx of fluid from the extracellular space. Currently there are two major concepts concerning intracellular osmolarity. If one accepts the idea

of an intracellular osmolarity considerably higher than that of the extracellular fluid (heart muscle, isotonic with .3 M sodium chloride) a rapid influx of fluid is easily explained.<sup>31</sup> This, of course, assumes either a ready permeability of the sarcolemma or an early and reversible loss of selective permeability in the absence of recognizable morphologic alteration and the continued utilization of energy by other components of the cell. Conway,<sup>32</sup> on the other hand, has expressed the belief that the intracellular and extracellular compartments are iso-osmolar, and that the differences observed by others were due to the breakdown of phosphocreatine and other energy-yielding compounds to form more particles, thus increasing the intracellular osmolarity. In the first instance, energy is utilized to maintain an osmotic gradient; in the second, reconstitution of metabolic products is necessary. This breakdown and subsequent freezing point depression take place rapidly at 0°C. The observations made here do not aid in resolving the problem since the muscle in a low state of activity (excised and incubated control) showed little alteration, and the muscle in a state of increased activity (fibrillation) showed maximal alteration. In the first case little breakdown took place, or sufficient energy was available to maintain the concentration gradient. In the case of fibrillation, increased activity necessitated maximal utilization of energy sources and rapid production of many molecules. Thus insufficient energy was present to maintain concentration gradients resulting from excessive utilization by other cellular components.

The continuation of the plasma membrane with the endoplasmic reticulum in the region of the "Z" bands (Fig. 2) provides a mechanism by which the difference in potential between the sarcoplasm and intercellular space may be rapidly conducted laterally to all portions of a given cell. This is in agreement with evidence put forth that local contraction within a single cell may be initiated only in the region of the "I" band, and that the contraction is propagated for a short distance into the cell.<sup>33</sup>

The sarcolemma was maintained in a relatively normal morphologic state during the first 3 to 4 hours of swelling. At about the time that the mitochondria ruptured, the sarcolemma also was disrupted. Examination of intact portions of the sarcolemma in 4 hour preparations showed normal structure, and the disruption was thought to be due to simple cellular swelling. With bursting, the pressure on the sarcolemma was eliminated and cytoplasmic components freed. The more soluble elements appeared in plasma while those less soluble were probably destroyed *in situ*. Blood enzyme values in these rabbits were elevated, presumably because of the large amount of skeletal

muscle traumatized during the surgical procedures. The PAS reaction of the sarcolemma remained unchanged throughout the period of investigation.

Cardiac glycogen is claimed to be present in acid soluble and acid insoluble forms. Bloom demonstrated a 93 per cent reduction of acid soluble and a 75 per cent reduction of acid insoluble glycogen following anoxia for 4 minutes.<sup>6</sup> For this reason it was felt that the glycogen granules, which disappeared promptly in the ischemic myocardium, probably represented acid soluble or unbound glycogen. So far we have not found any particles corresponding to the glycogen accumulations at the "A," "I," and "Z" bands described by Studnitz<sup>84</sup> and Dempsey.<sup>85</sup>

Marked clumping of nucleoplasm occurred before very extensive cytoplasmic changes had taken place. The alteration consisted primarily of aggregation of nuclear substance and some tendency toward accumulation near the nuclear envelope. Associated with the clumping was a loss of density in many regions. Clumping of a similar nature had been encountered in cells fixed in acid solutions or those of high sodium chloride concentration (.9 per cent), in virus-infected cells and in cells which were unfixed for a period sufficient to permit a reduction of the pH of a .01 molar phosphate buffer to a point below 7. There are undoubtedly many other circumstances that can bring about nuclear clumping. Since this feature repeatedly occurs with little cytoplasmic alteration one may assume that excessive penetration of extracellular sodium ions or loss of intracellular potassium ions is not of paramount importance. In view of the rapid change in glycogen content, a shift in pH or an alteration of cytoplasmic osmolarity might affect the nucleus. Muscle cells are not well suited to solve this problem since they have a large component of highly stable cytoplasmic substance. For instance, mild pH or ionic shifts would not be expected to alter the myofilaments.

The mitochondrial alterations were those which might be expected in an "osmometer" placed in hypotonic solution. The earliest evidences of mitochondrial swelling appeared in 20 to 30 minutes and progressed for  $3\frac{1}{2}$  to 4 hours, at which time disruption and collapse began. Not all mitochondria proceeded through the complete cycle. Since swelling occurred so early and was accompanied by minimal cytoplasmic changes, one is faced with the same problem encountered in attempting to explain cellular swelling. However, in this instance there are a number of additional features. The mitochondrial swelling does not commence until the ischemia has existed for 20 or more minutes; from this point on it parallels cellular swelling. Moreover, swelling



of the mitochondria was proportional to the cytoplasmic alteration in the fibrillating heart. Thus, the mitochondrial alterations began at about the time the myofibrils lost their ability to contract, or at the time when there was no further energy available for contraction. It appears, therefore, that mitochondrial form is maintained in relation to evidences of available energy. It would seem that the source of energy for maintenance of mitochondrial integrity is related to that necessary for muscular contraction.

A striking feature was the existence of contraction bands at the "Z" region in all specimens of ischemic muscle at all periods up to 20 minutes after ligation; after 30 minutes the contraction bands were absent and did not return. The band was attributed to the irritative effects of the fixative on viable muscle. Its absence and the appearance of a distinct "I" band were thought to result either from an inability of fibrils to contract, or a loss of the energy necessary for contraction.

The spatial relationship of myofilaments to each other became more clearly manifest in lesions of longer duration. This probably resulted from loss of substance as well as density with stretching of muscle. Groups of myofilaments exhibited splitting, but the aggregates that remained showed no appreciable alteration of the hexagonal packing observed on cross section.

Ring<sup>29</sup> had shown that the extent of necrotic tissue 5 days after coronary ligation did not necessarily coincide with the initial distribution of blanching and cyanosis observed after coronary ligation and suggested that a minimum of 5 days was necessary to determine the extent of injury. In each instance, however, he noted a very sharp line of demarcation between damaged and normal muscle. In the rabbit hearts we examined, a similar line of demarcation was apparent; that the sharpness was related to the distribution of the blood supply was self evident. The exactness of demarcation could best be related to a functional end-artery status. The intermixture of intact and necrotic fibers at 5 days and the persistence of intact muscle in the fibrous scar at 6 weeks suggests the existence or the development of sufficient circulation to maintain their viability. This was brought out in electron micrographs of muscle fibers lying within the ischemic area in which variable degrees of damage were evident. Barely sufficient circulation may be augmented by varying degrees of muscular activity. Both light and electron microscopy demonstrated the preservation of a subendocardial zone. This has been a common observation and has been attributed to diffusion of nutrients from the cardiac chamber or the flow of blood through thebesian veins.

Endothelial cells have been considered to be sensitive to oxygen deficit; indeed, they exhibit alterations similar to those observed in muscle although the alterations develop at a slower rate. The energy expenditure of endothelium is less than that of muscle, a factor which may well explain the discrepancy in rate of degeneration.

Specimens of heart incubated for 3 hours at 37° in .01 M phosphate buffer, pH 7.4, showed only slight alteration in contrast to the lesions encountered in ischemic myocardium. The difference was probably related to the failure of contraction in the excised fragment; the ischemic muscle continued to contract and use energy for varying periods of time. The presence or absence of glucose in the incubating medium had little influence on the structure of the myocardium from the controls, indicating that exogenous sources of energy were not essential for periods as long as 3 hours.

#### SUMMARY

The premise that chemical alterations in experimentally induced ischemia of the myocardium are associated with morphologic alterations was substantiated. As early as 5 minutes after coronary arterial ligation in rabbits there was disappearance of glycogen from ischemic myocardium; simultaneously, structural changes became manifest. This was characterized by clumping of nucleoplasm and cytoplasmic distortion. Ischemia continuing for 30 minutes was sufficient to result in loss of contractile capacity and cell death.

The actual disintegration of muscle fibers proceeded at a varied rate, seemingly dependent in part upon the activity of the muscle during the period of ischemia.

Electron microscopy clearly demonstrated continuity of the sarcolemmal membrane of heart muscle and its endoplasmic reticulum. This was thought to provide a morphologic basis for the conduction of surface potentials throughout the cardiac muscle fiber.

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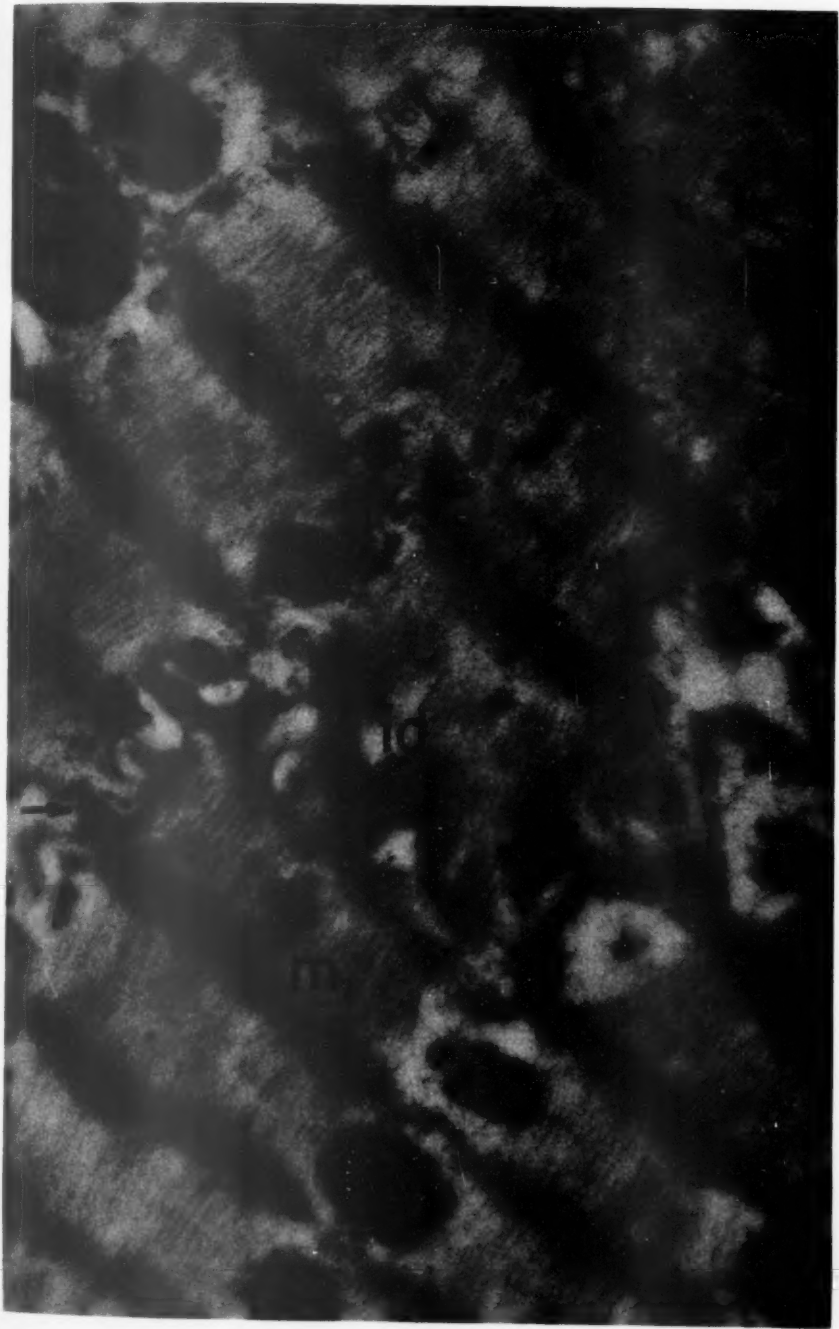
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[ Illustrations follow ]

## LEGENDS FOR FIGURES

- FIG. 1. Normal rabbit myocardium. A prominent intercalary disc (id) is continuous with the cell membrane (arrow). This relationship holds at the right margin as well. Elements of the endoplasmic reticulum (er) are particularly evident in the region of contraction bands (cb). Mitochondria (m) have a linear arrangement between bundles of myofilaments. A tangential section of a mitochondrion is present at  $m_1$ . There are two lipid bodies visible adjacent to the mitochondrion in the lower right corner.  $\times 50,000$ .



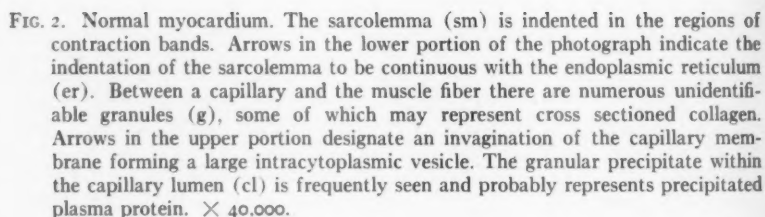


FIG. 2. Normal myocardium. The sarcolemma (sm) is indented in the regions of contraction bands. Arrows in the lower portion of the photograph indicate the indentation of the sarcolemma to be continuous with the endoplasmic reticulum (er). Between a capillary and the muscle fiber there are numerous unidentifiable granules (g), some of which may represent cross sectioned collagen. Arrows in the upper portion designate an invagination of the capillary membrane forming a large intracytoplasmic vesicle. The granular precipitate within the capillary lumen (cl) is frequently seen and probably represents precipitated plasma protein.  $\times 40,000$ .

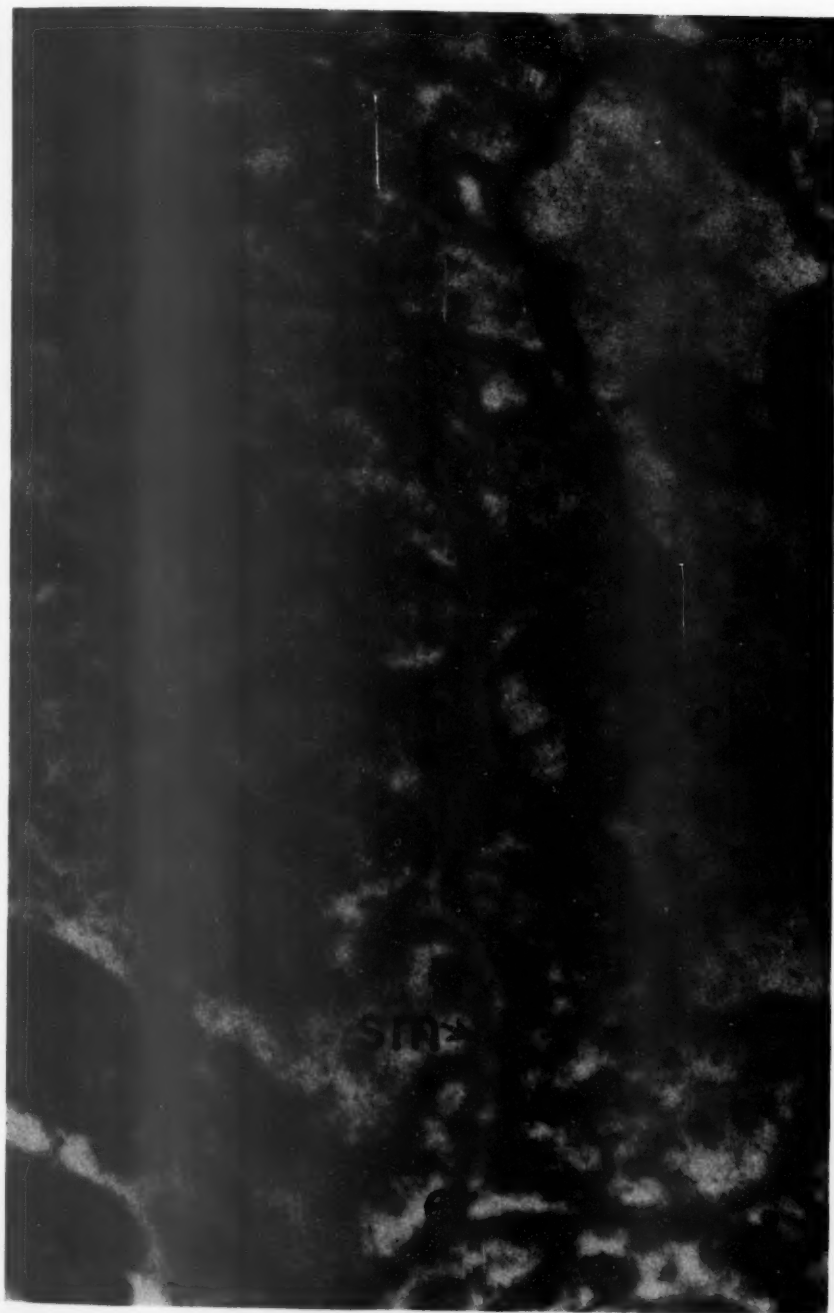
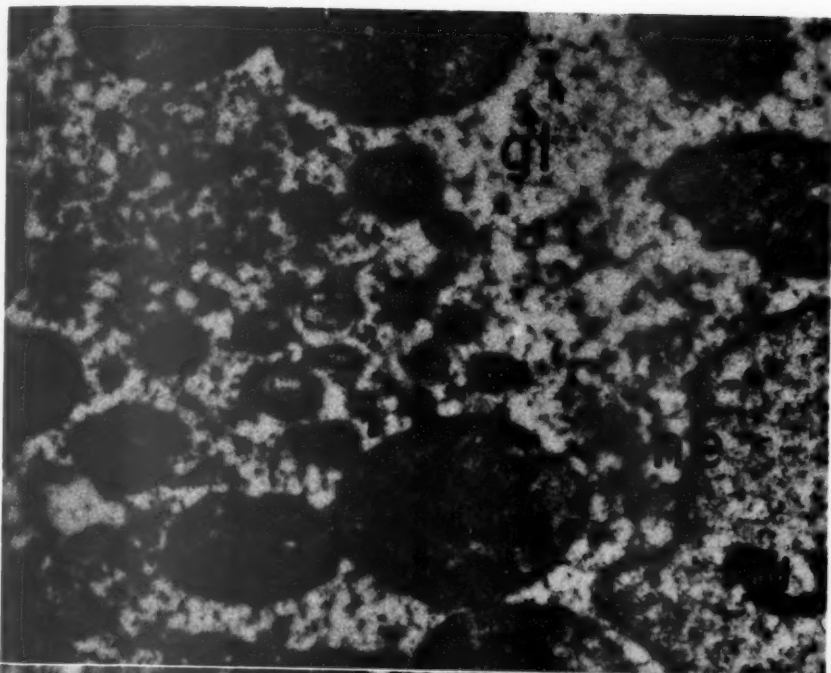


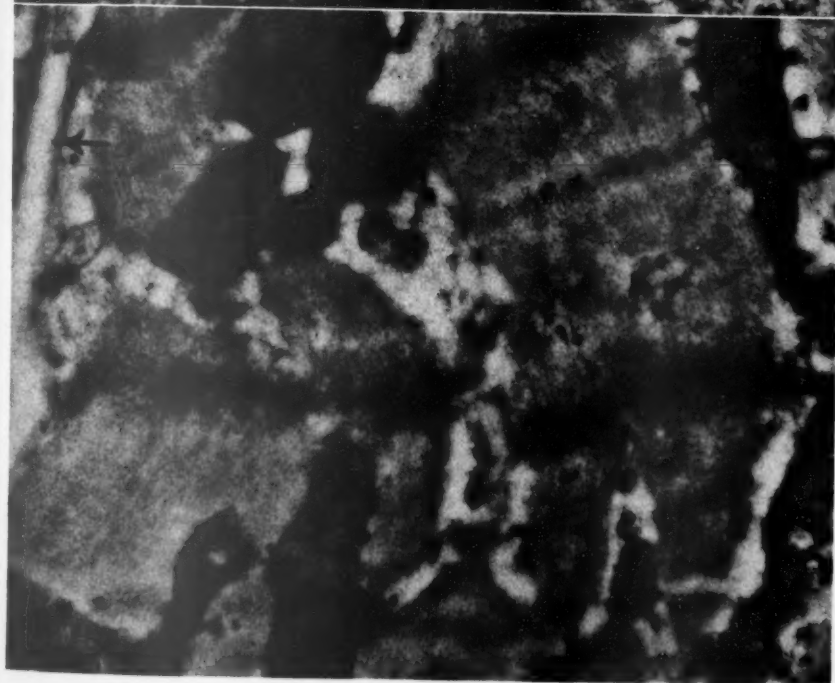
FIG. 3. Normal myocardium. Glycogen granules (gl) are clearly visible in the perinuclear region. A segment of endoplasmic reticulum with ribonucleic acid granules is indicated by the arrows. The smooth vesicular structures are present in random arrangement in perinuclear location. The nuclear envelope (ne) encloses a mass of evenly distributed granular and fibrillar substance. The nucleolus (nu) is much more dense than the nucleoplasm.  $\times 23,000$ .

FIG. 4. Myocardial ischemia, 5 minutes. Clear spaces are evident around unaltered mitochondria and between the myofibrils. The space between two cells (arrow) is not increased appreciably. A small segment of nucleus may be seen (n).  $\times 23,000$ .





3



4

FIG. 5. Myocardial ischemia, 5 minutes. Perinuclear glycogen has disappeared. The nucleoplasm (n) shows clumping and margination. Endoplasmic reticulum (er) is normal at this stage and contraction bands are visible.  $\times 50,000$ .






FIG. 6. Myocardial ischemia, 10 minutes. Segments of muscle and endothelium (c) are visible. Numerous small vesicles appear near the cytoplasmic membrane (arrows) of each cell type. There is some clumping of the endothelial nucleoplasm (n). The attachment of the sarcolemma at the "Z" band region is shown. Alterations of the muscle nucleus are similar to those seen in endothelium, but are of a more advanced nature.  $\times 40,000$ .

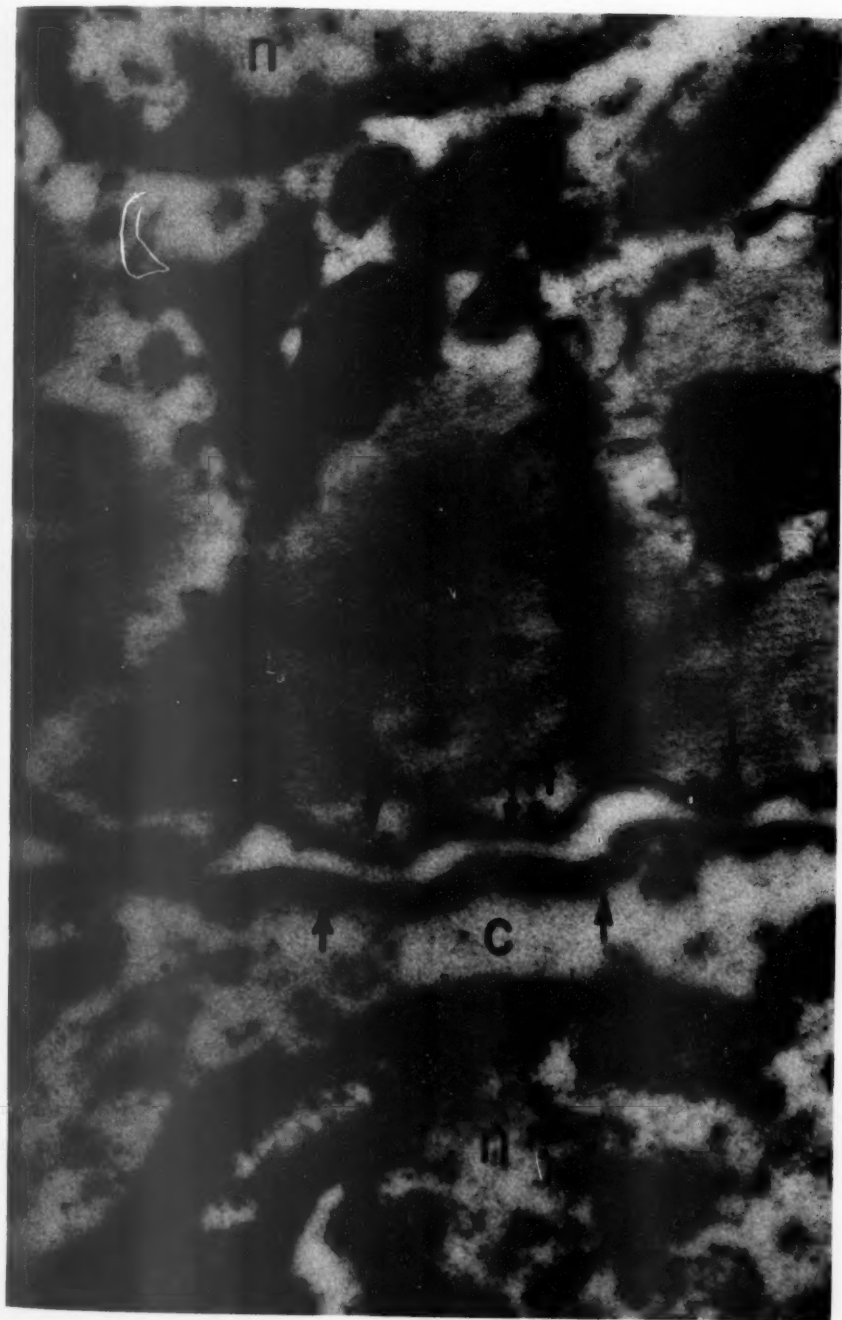


FIG. 7. Myocardial ischemia, 20 minutes. There is little more than progression of the intracellular swelling seen in figures 4 to 6. Mitochondria retain normal appearance. A portion of an erythrocyte may be seen (rc).  $\times 23,000$ .





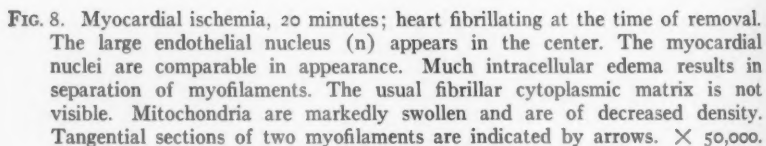
The image is an electron micrograph showing a cross-section of myocardial tissue. A large, dark, oval-shaped endothelial nucleus is visible in the center. The surrounding cytoplasm is filled with various organelles, including mitochondria that appear swollen and less dense than normal. The myofibrils are separated by a wide space, indicating edema. Two arrows point to tangential sections of myofilaments. The overall texture is grainy, typical of electron microscopy.

FIG. 8. Myocardial ischemia, 20 minutes; heart fibrillating at the time of removal. The large endothelial nucleus (n) appears in the center. The myocardial nuclei are comparable in appearance. Much intracellular edema results in separation of myofilaments. The usual fibrillar cytoplasmic matrix is not visible. Mitochondria are markedly swollen and are of decreased density. Tangential sections of two myofilaments are indicated by arrows.  $\times 50,000$ .

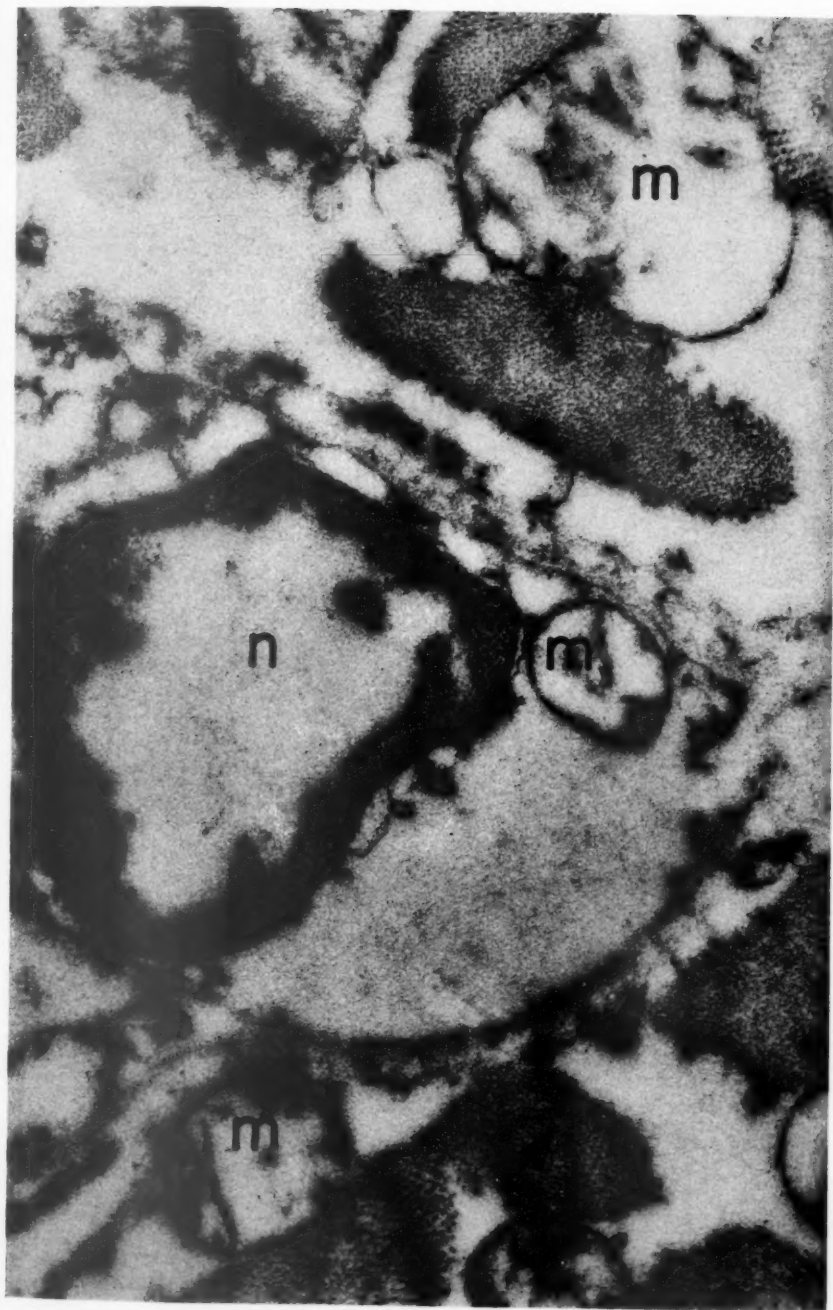


FIG. 9. Myocardial ischemia, 35 minutes. Nuclear and cytoplasmic alterations are in more advanced state. Myofibrils lack contraction bands, and an "I" band is present. Mitochondria are swollen, dark bodies (db) appear within them and the spatial arrangement of their cristae is disturbed.  $\times 50,000$ .



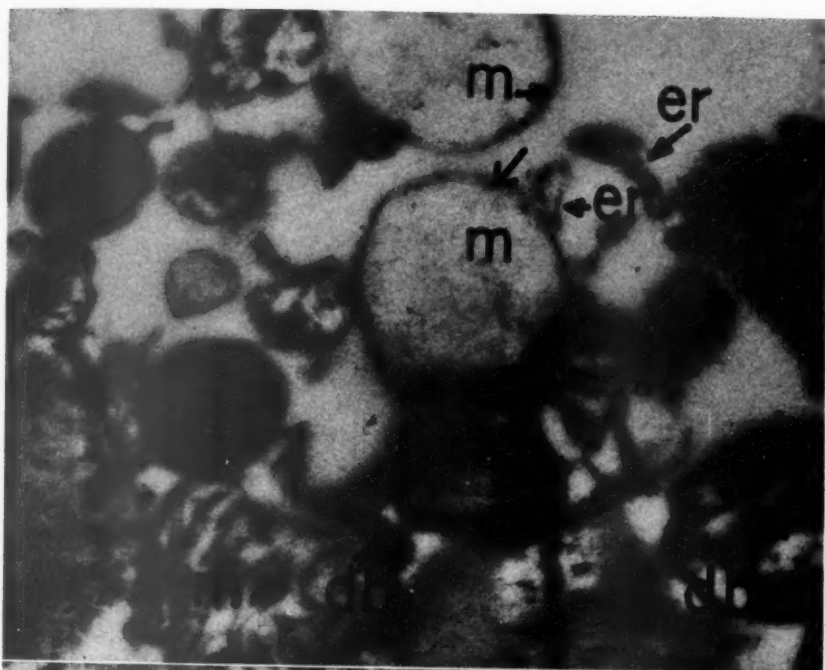
FIG. 10. Myocardial ischemia, one hour. The intercalary disc (id) shows no evidence of separation but clear areas are evident adjacent to it. These are in continuity with the spaces between myofibrils and apparently represent a similar process. Mitochondria resemble those seen in Figure 9.  $\times 28,000$ .



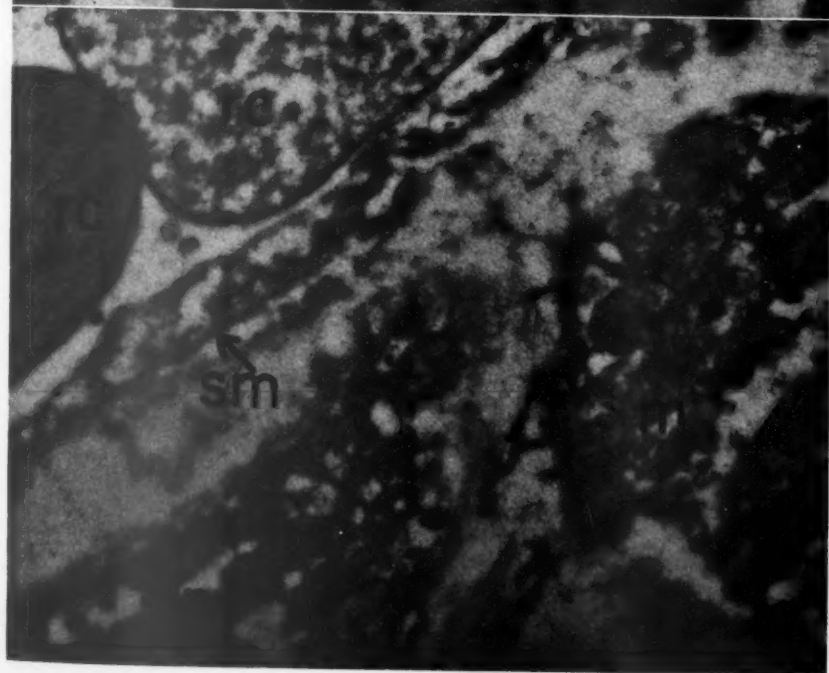
FIG. 11. Myocardial ischemia, 4 hours. There is disruption of mitochondria (m) and loss of their content. Swollen endoplasmic reticulum (er) is common at this stage.  $\times 35,000$ .

FIG. 12. Myocardial ischemia, 5 hours. Two red cells (rc) show very different degrees of hemoglobin loss. There is continued progression of the degenerative changes in muscle. Loss of continuity of the sarcolemmal membrane is now visible although the fibers still maintain their relations and organelles are not extruded.  $\times 16,800$ .





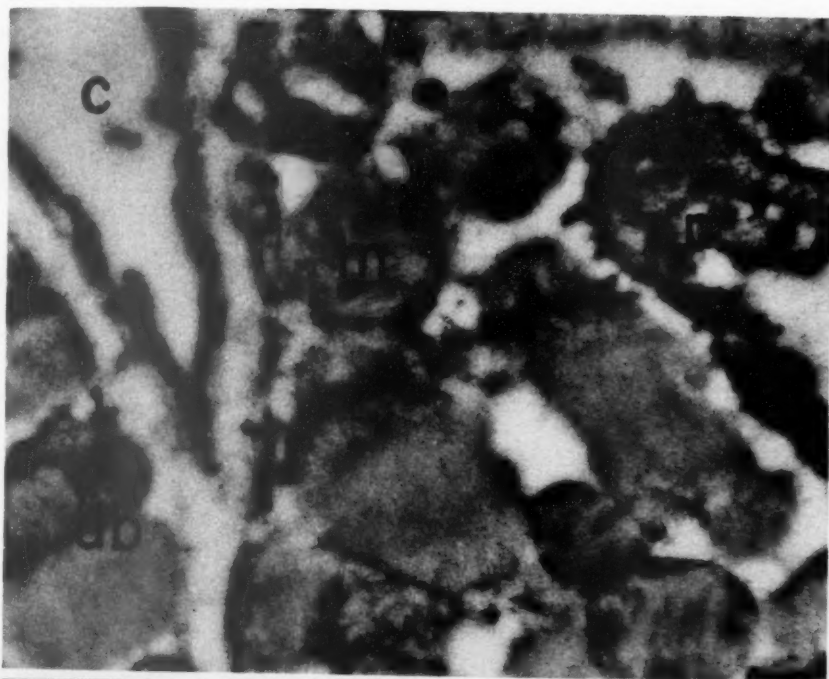
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FIG. 13. Myocardial ischemia, 22 hours. Large numbers of dense bodies (db) are visible both inside and outside of mitochondria. Despite progression of alterations no new features are noteworthy.  $\times 23,000$ .

FIG. 14. Normal heart incubated at  $37^{\circ}\text{C}$ . for 3 hours in .01 M Krebs-Ringer phosphate buffer. There is only slight edema of the muscle fiber and minor alterations of the mitochondria. Contraction bands (cb) are maintained. The endoplasmic reticulum appears normal. The nucleus, not illustrated, shows some margination of nucleoplasm.  $\times 50,000$ .



13



14



## SENSITIZATION BY POTASSIUM DEFICIENCY FOR THE PRODUCTION OF MYOCARDIAL NECROSIS BY STRESS\*

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Combined treatment with certain corticoids and so-called "sensitizing Na-salts" (e.g., sodium phosphate, sodium perchlorate, disodium sulphate, but not sodium chloride) produces infarct-like patches of myocardial necrosis in various experimental animals. This "Electrolyte-Steroid-Cardiopathy with Necrosis" (ESCN) is prevented by several chlorides and with particular ease by magnesium chloride or potassium chloride. In corticoid-conditioned rats, similar foci of multiple myocardial necrosis are produced by a variety of forms of stress (e.g., electric shock, forced muscular exercise, cold, heat, and various drugs); these stress-induced necroses are also prevented by magnesium chloride and potassium chloride.<sup>1</sup> Thus, there may be some common pathway in the mechanism through which diverse agents influence the cardiac muscle.

In view of the protective effect of potassium and magnesium, the question arose whether a dietary deficiency of these elements could predispose the heart to the induction of necrosis by various agents. Experiments performed to determine this point showed that magnesium or potassium deficient diets greatly sensitized the rat to myocardial necrosis induced by the intravenous injection of a protease (papain extract). Curiously, the sensitizing effects of both the magnesium and the potassium deficient diets could be abolished by supplements of either magnesium chloride or potassium chloride.<sup>2</sup> It was found, furthermore, that when rats were maintained on a potassium deficient ration containing mere maintenance levels of magnesium, then sodium phosphate, sodium perchlorate or disodium sulphate (unlike equivalent amounts of sodium chloride) rapidly provoked severe cardiac necrosis before the diet itself resulted in any obvious morbid alteration. The very severe lesions induced by these "sensitizing Na-salts," in animals on this diet, could also be prevented by the administration of either potassium chloride or magnesium chloride.<sup>3,4</sup>

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The object of this communication is to describe experiments showing that cardiac muscle is selectively sensitized to the induction of necrosis by various agents in rats maintained on the same potassium deficient diet for only one week. The diet duplicates the "selective conditioning" to the normally latent cardiotoxic effects of stress. This has previously been found to occur after pretreatment with certain corticoids.

#### METHODS

Two hundred thirty female Sprague-Dawley rats, with an average initial body weight of 52 gm. (range, 50 to 56 gm.), were subdivided into 23 equal groups and treated as indicated in Table I.

TABLE I  
*Effect of Potassium Deficiency  
Upon the Potential Cardiotoxic Action of Various Agents*

| Group | Treatment                         | Mean incidence<br>of cardiac necrosis | Mortality<br>(%) |
|-------|-----------------------------------|---------------------------------------|------------------|
| 1     | K-deficiency                      | 0                                     | 0                |
| 2     | Noradrenaline                     | $0.4 \pm 0.23^*$                      | 0                |
| 3     | Noradrenaline + K-deficiency      | $1.6 \pm 0.38$                        | 30               |
| 4     | Vasopressin                       | 0                                     | 0                |
| 5     | Vasopressin + K-deficiency        | $1.7 \pm 0.30$                        | 20               |
| 6     | Thyroxine                         | 0                                     | 0                |
| 7     | Thyroxine + K-deficiency          | $0.3 \pm 0.15$                        | 30               |
| 8     | Dihydrotachysterol                | 0                                     | 0                |
| 9     | Dihydrotachysterol + K-deficiency | $2.0 \pm 0.44$                        | 10               |
| 10    | Plasmocid                         | $0.1 \pm 0.10$                        | 0                |
| 11    | Plasmocid + K-deficiency          | $1.3 \pm 0.20$                        | 100              |
| 12    | Restraint                         | 0                                     | 10               |
| 13    | Restraint + K-deficiency          | $2.4 \pm 0.34$                        | 30               |
| 14    | Cold                              | 0                                     | 0                |
| 15    | Cold + K-deficiency               | $2.1 \pm 0.30$                        | 60               |
| 16    | Heat                              | $0.1 \pm 0.10$                        | 30               |
| 17    | Heat + K-deficiency               | $1.0 \pm 0.20$                        | 10               |
| 18    | Vagotomy                          | $0.2 \pm 0.17$                        | 80               |
| 19    | Vagotomy + K-deficiency           | $1.2 \pm 0.39$                        | 0                |
| 20    | Quadriplegia                      | 0                                     | 0                |
| 21    | Quadriplegia + K-deficiency       | $1.0 \pm 0.27$                        | 0                |
| 22    | Trauma                            | 0                                     | 0                |
| 23    | Trauma + K-deficiency             | $0.7 \pm 0.20$                        | 10               |

\*  $\pm$  indicates standard errors of the mean.

The composition of the potassium deficient diet (supplied by Nutritional Biochemicals Corporation, Cleveland, Ohio) was as follows:

*Chief Ingredients (per cent)*

|                         |      |
|-------------------------|------|
| Corn starch .....       | 64.2 |
| Casein .....            | 30.0 |
| Butterfat .....         | 3.5  |
| Calcium carbonate ..... | 1.3  |
| Sodium chloride .....   | 1.0  |

*Supplements (gm. per 100 pounds of diet)*

|  |         |
|--|---------|
| Vitamin A concentrate<br>(200,000 units per gm.) ..... | 4.5     |
| Vitamin D concentrate<br>(400,000 units per gm.) ..... | 0.25    |
| Alpha tocopherol .....                                 | 5.0     |
| Ascorbic acid .....                                    | 45.0    |
| Inositol .....   | 5.0     |
| Choline chloride .....                                 | 75.0    |
| Menadione .....  | 2.25    |
| p-Aminobenzoic acid .....                              | 5.0     |
| Niacin .....   | 4.5     |
| Riboflavin .....                                       | 1.0     |
| Pyridoxine HCl .....                                   | 1.0     |
| Thiamine HCl .....                                     | 1.0     |
| Calcium pantothenate .....                             | 3.0     |
| Biotin .....   | 0.020   |
| Folic acid .....                                       | 0.090   |
| Vitamin B-12 .....                                     | 0.00135 |

This diet was administered to all the odd-numbered groups listed in Table I, during the 8 days of the experiment. The even-numbered groups were kept on Purina Fox Chow (Ralston Purina Company of Canada), but the amount of their food was so reduced as to keep the body weight approximately equal to that of the animals on the potassium deficient diet. In this manner, it was hoped to eliminate any possible nonspecific effect of malnutrition which might have been related to the fact that potassium-deficient animals do not grow at the normal rate.

The potentially cardiotoxic agents used were the following:

*Noradrenaline* (Sterling-Winthrop Research Institute), 200 µg. in



0.2 ml. of oil, administered subcutaneously, twice on the sixth day.

*Vasopressin* (Parke, Davis & Company, Ltd.), 10 I.U. in 0.5 ml. of water, introduced subcutaneously, twice on the sixth day.

*Thyroxine* (British Drug Houses, Ltd.), 125  $\mu$ g. in 0.2 ml. of water, given subcutaneously, daily, throughout the experiment.

*Dihydrotachysterol* (Wander), 25  $\mu$ g. in 0.2 ml. of water, administered by stomach tube, daily, throughout the experiment.

*Plasmocid* [8-(3-3-Diethylaminopropylamino)-6-methoxyquinoline]; (The Lilly Research Laboratories), 0.4 mg. in 0.2 ml. of water, injected intraperitoneally, 3 times during the sixth day.

*Restraint.* The animals were strapped to wooden boards with adhesive tape in the prone position for a period of 17 hours on the sixth day.

*Cold.* The rats were immersed in icy water for two periods of 3.5 minutes on the sixth day.

*Heat.* Immersion was carried out in water at 48° C. for two periods of 3.5 minutes on the sixth day.

*Vagotomy.* Bilateral transection of the vagus nerves was performed on the sixth day. As in all surgical procedures, this was carried out under ether anesthesia.

*Quadriplegia.* The motor nerves of all 4 extremities (sciatic, femoral and obturator nerves, brachial plexuses) were severed, thus depriving the rat of the use of its limbs.

*Trauma.* The stomach, ileum and cecum were crushed—3 times each—with a hemostat.

All the surviving rats were sacrificed on the eighth day. In each instance, the heart was fixed in neutral formalin and embedded in paraffin. Calcium was demonstrated histologically by the von Kóssa method. The severity of the cardiac lesions was graded by an arbitrary scale of 0 to 3. The mean incidence of the lesions (with standard errors) and the mortality rates are listed in Table I.

## RESULTS

It is evident from the data in Table I that even the comparatively mild nutritional deficiency induced by the low potassium diet for one week greatly sensitized the heart to the necrosis-producing effects of various agents. This was true in the case of substances (e.g., noradrenaline, vasopressin, dihydrotachysterol and plasmocid) that, at higher dose levels, could produce cardiac lesions in animals on normal diets.<sup>1</sup> It also occurred as the result of various forms of stress (e.g., restraint, cold, heat, vagotomy, quadriplegia or intestinal trauma) which normally exerted no specific cardiotoxic effects.

Histologically, the lesions were rather similar to those designated ESCN which were induced by combined treatment with certain electrolytes and steroids. Irrespective of the precipitating agent used, the cardiac lesions in the potassium deficient rats consisted of more or less extensive patches of necrosis, distributed throughout the heart, but with particular frequency in the subendocardial layers, the papillary muscles, and the auricles. The necrotic muscle fibers were occasionally impregnated with calcium, but usually the debris derived from them was removed by histiocytes, and eventually they were replaced by scar tissue. Often, Anitschkow myocytes ("caterpillar cells") and multinucleated giant cells appeared in the affected regions. In extreme lesions, the contractile elements disappeared completely from large portions of the ventricular wall. In the auricles, in these instances, only occasional muscle fibers remained (Figs. 1 to 7). As an incidental observation, it was noted that at the low-dose level at which it was given, dihydrotachysterol failed to produce any renal calcification in the control rats. On the other hand, in the rats given the potassium deficient diet, its administration resulted in intense nephrocalcinosis. Since the brief period of potassium deficiency itself produced no such renal lesion, it was concluded that dietary potassium deprivation sensitized the kidney to the production of nephrocalcinosis by dihydrotachysterol (Fig. 8).

#### DISCUSSION

In evaluating these data, it should be kept in mind that the "low-K diet" prepared by the Nutritional Biochemicals Corporation, used in these and previously reported investigations, is also comparatively poor in magnesium. It was found to contain 52 mg. of magnesium per kg. This is close to the minimum amount necessary for growth, according to Tufts and Greenberg.<sup>5</sup> Since both potassium and magnesium salts exert a protective effect against the ESCN phenomenon, it is possible that the relative poverty of magnesium also played a sensitizing role in the present experiments. In any event, it is clear that one week on the deficient diet sufficed to render rats unusually sensitive to the induction of massive myocardial necrosis by a great variety of agents. These agents apparently had as their only feature a capacity to produce stress.

A possible relationship between true cardiac infarcts in man and the ESCN phenomenon has been discussed elsewhere.<sup>1</sup> It is re-emphasized here that the cardiac lesions elicited by various agents in rats on low potassium diet in the present experiments resemble the ESCN in their histologic characteristics and their distribution within the heart. There is, moreover, no evidence of morphologic alteration

in the coronary vessels. Nonspecific systemic stress can elicit such myocardial lesions after conditioning with certain corticoids<sup>1</sup> and following maintenance during a brief period on a potassium deficient diet. It remains to be seen whether this type of cardiopathy has any equivalent in human disease. Our experiments merely indicate that, following suitable dietary conditioning, various stressors may precipitate myocardial necrosis in rats.

#### SUMMARY

In rats maintained for one week on a potassium deficient diet, the myocardium showed no detectable structural alterations. This diet also contained only minimum maintenance levels of magnesium. However, in similar animals, extensive myocardial necrosis was induced by a variety of factors which were ineffective in causing this lesion in animals on normal diet. The factors utilized were noradrenaline, vasopressin, thyroxine, dihydrotachysterol, plasmocid, forced restraint, cold or hot baths, vagotomy, quadriplegia and intestinal trauma. It is concluded that a brief period of nutritional deficiency can "selectively condition" the myocardium to the cardiotoxic effect of various forms of stress.

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*[ Illustrations follow ]*

## LEGENDS FOR FIGURES

All illustrations are of sections stained by the von Kóssa method.

- FIG. 1. General view of the heart of a rat exposed to forced restraint while on the low potassium diet (group 13). The dark-staining areas in the left auricle, sub-endocardial region of the left ventricle and the cranial portion of the right ventricle contain calcified, necrotic muscle.  $\times 10$ .
- FIG. 2. Wall of the left auricle shown in Figure 1. The photograph represents the zone between normal muscular trabeculae (bottom) and septums in which the muscle is replaced by connective tissue and histiocytes (top). The jet-black area near the upper edge of the photograph is calcified.  $\times 100$ .
- FIG. 3. Right auricular appendage of the heart shown in Figure 1. Upper right: Atrophic septums, in which the muscle tissue has almost completely disappeared, except for a small light-staining band near the center of the field.  $\times 100$ . Lower left: The light-staining band composed of fairly normal muscle is shown at higher magnification.  $\times 420$ .
- FIG. 4. The left auricular appendage of a control rat (group 12) to show the normal thickness and muscularity of the septums.  $\times 100$ .

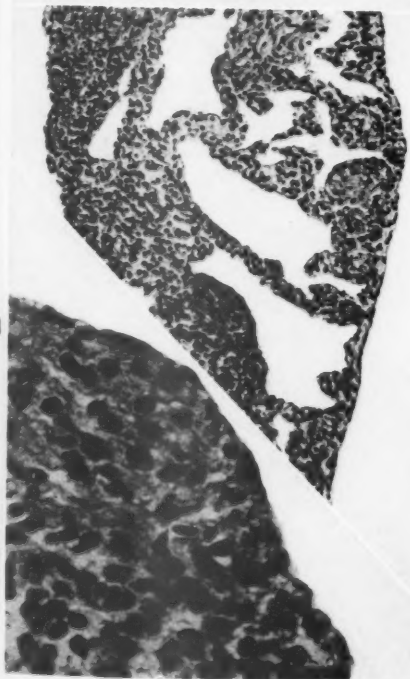
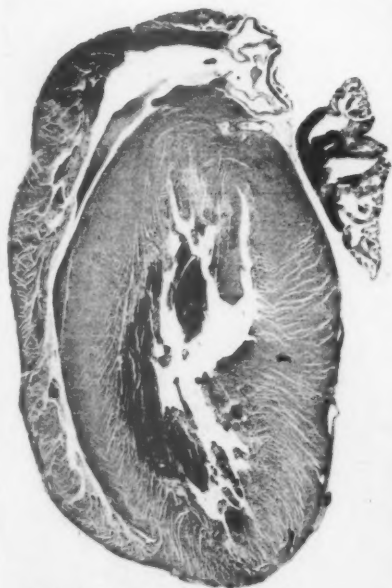


FIG. 5. Section through the entire thickness of the left ventricle of a rat kept on the low potassium diet and treated with dihydrotachysterol (group 9). Widespread calcification and necrosis of muscle tissue and some edema (light halo) appears around the arteries.  $\times 35$ .

FIG. 6. A subendocardial focus, from which the muscle tissue has almost completely disappeared, following vasopressin treatment of a rat kept on the potassium deficient diet (group 5).  $\times 100$ .

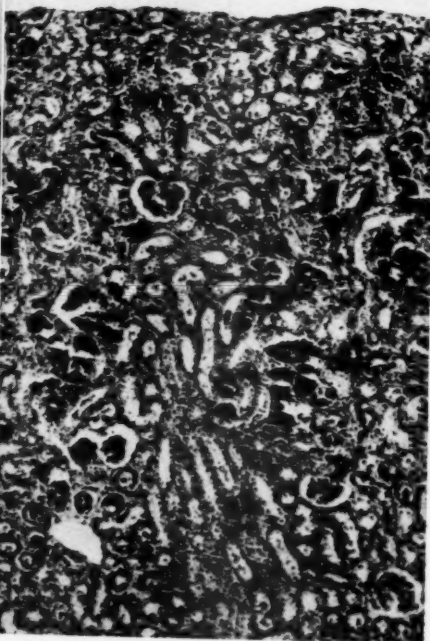
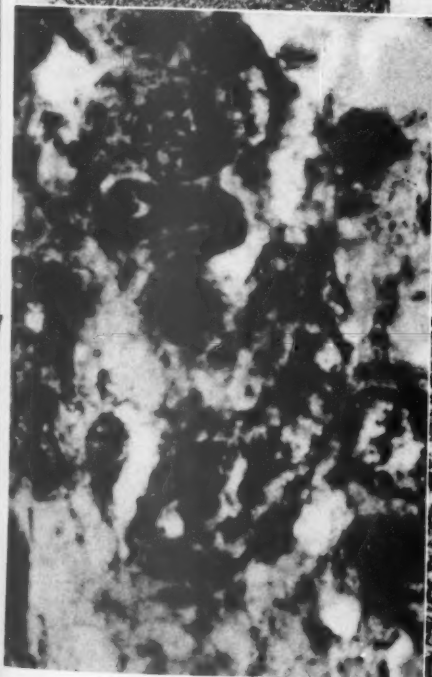
FIG. 7. Higher magnification of a typical "caterpillar cell," just below the middle of the left edge of the picture. Multinucleated giant cells are seen in the remainder of the field (group 13).  $\times 1,140$ .

FIG. 8. Widespread calcification of the renal cortex in a rat on the potassium deficient diet and treated with dihydrotachysterol (group 9).  $\times 75$ .

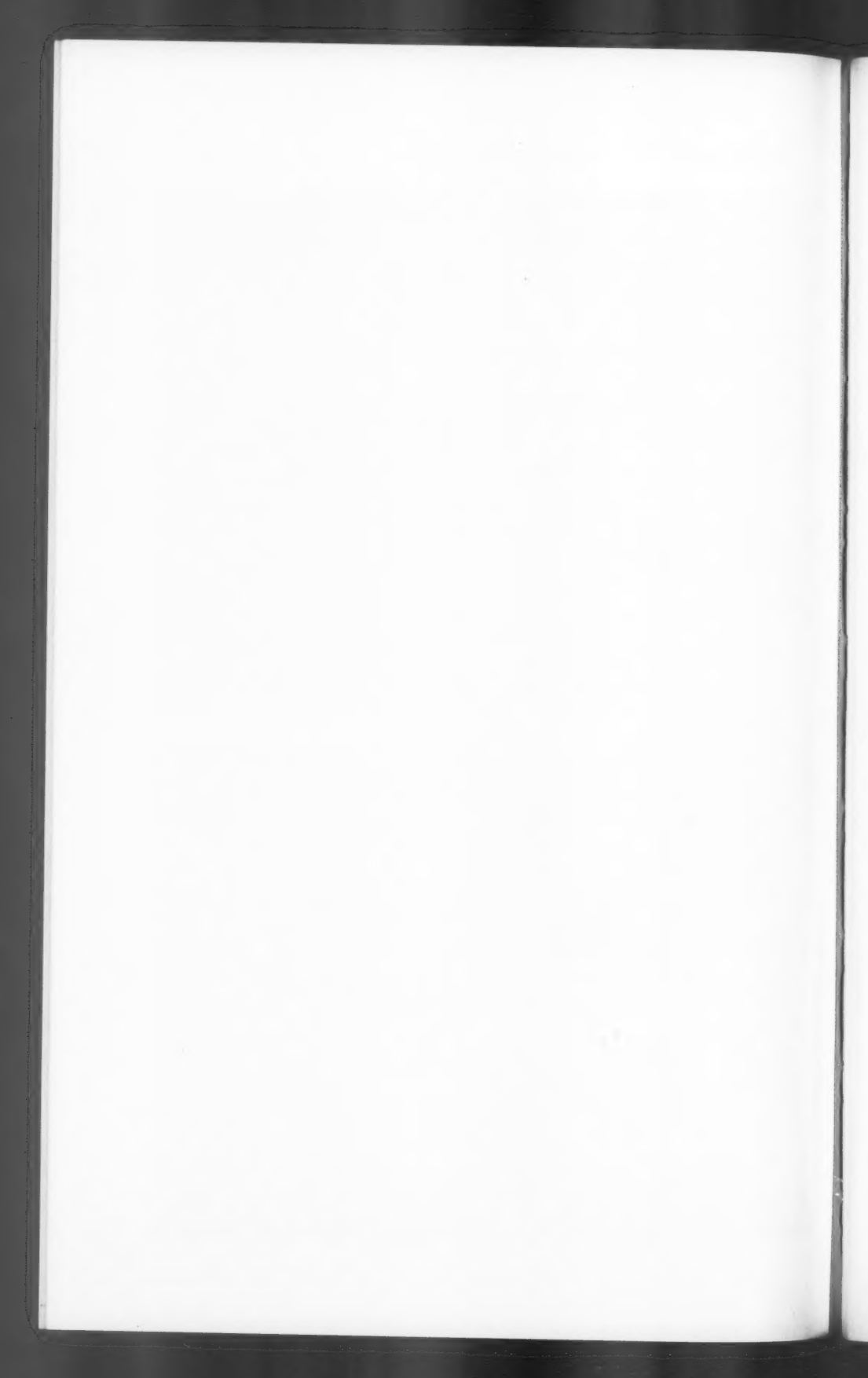




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## HYPERADRENOCORTICISM (CUSHING'S DISEASE): A STUDY OF SURGICALLY RESECTED ADRENAL GLANDS\*

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The problem of relating structural alterations in the adrenal cortex to functional changes occurring in a variety of syndromes is of interest to both the pathologist and the clinician. In general, the relationship of adrenal cortical atrophy or destruction to the clinical syndrome of hypoadrenocorticism is clear. On the other hand, the recognition of the unusually stimulated cortex rests upon marked changes in weight, advanced hyperplasia, adenomas, and carcinomas; few more refined morphologic criteria are available. In reported cases of typical Cushing's disease, few are cited in which the adrenal glands are normal in size or only equivocally enlarged.<sup>1,2</sup> Twenty per cent of the cases in the present group would ordinarily be included in this category. One might conclude that in some instances of Cushing's disease the abnormality lies elsewhere than in the adrenal gland, or that there may not always be a morphologic reflection of the hyperfunctioning state. An alternative possibility—and perhaps a more hopeful one for the pathologist—is that the adrenal gland lesions customarily related to hyperactivity represent extremes and that subtle alterations are often overlooked. The present investigation was initiated as a histologic study of benign cortical hyperplasia in Cushing's disease, in the hope of elucidating the early or milder alterations and of tracing their progression to the fully developed state. Cases of adrenal adenoma associated with Cushing's disease were included for the purpose of comparison.

### MATERIAL AND METHODS

Hyperactive glands procured from 20 patients with Cushing's syndrome were examined. There were 16 pairs of glands removed by subtotal (approximately 90 per cent) resection (one or two stage procedure). In 4 cases the specimen consisted of a single adrenal containing a large, apparently solitary nodule of the type usually

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classified as an adenoma. In the latter group, the opposite adrenal was inspected by the surgeon at the time of operation, and in two cases a biopsy specimen was obtained. From clinical and laboratory data, all of these patients were considered typical examples of Cushing's syndrome. There were 18 females and 2 males, ranging in age from 16 to 42 years. Many of the patients had received various forms of therapy with only transient or no improvement, and most patients had received several doses of cortisone in the 12 to 24 hour period before operation. It is unlikely that this immediate preoperative treatment had any significant effect on the structure of the adrenal cortex,<sup>3</sup> but unfortunately this assumption was not subject to control.

Control biopsy specimens were obtained from 14 patients during the course of retroperitoneal operations for renal cysts, stones, carcinoma, and tuberculosis. None of these patients had hypertension or endocrine disorders, and none were debilitated. Sufficient tissue was obtained in each case to permit the application of all the procedures utilized in the glands removed in Cushing's disease. Although all layers of the cortex were present in each specimen, one could not consider that each was necessarily representative of the whole gland.

Tissues were collected directly after removal, weighed, and placed in 10 per cent buffered formalin. They remained in fixative for varying periods of time before sectioning and staining. Several blocks of each gland were embedded in paraffin, sections were cut at 6  $\mu$ , and stained with hematoxylin and eosin. Multiple frozen sections, 15 to 20  $\mu$  in thickness were cut from each gland, often serially. These were stained as follows<sup>4</sup>: Sudan IV to stain the general group of sudanophilic lipids; the Schultze test to demonstrate cholesterol and similar sterols unsaturated at the 5:6 bond; the Windaus reaction, which depends on the precipitation of insoluble birefringent crystalline digitonides of cholesterol and other beta sterols; the Schiff reaction to indicate the presence of active aldehydic carbonyl groups; and the naphthoic acid hydrazide (NAH) reaction to demonstrate the presence of active ketonic and aldehydic carbonyl groups. The lipid nature of reacting substances was confirmed by control sections incubated in acetone and other organic solvents. No attempt was made to unmask possible non-reacting lipids. Care was taken in sectioning to represent as nearly as possible true cross sections of the gland.

The advantages and disadvantages of these methods and the significance of their interpretation have been fully discussed elsewhere.<sup>4,5</sup> Artifacts generated by standing and fixation probably influenced the final patterns. It is sufficient for this study to consider that the reac-

tions demonstrated somewhat different components of the cortical lipid. These have been utilized in an attempt to define the morphologic correlations of lipid variations in the cortex.

## RESULTS

### *Gross Observations*

Four pairs of adrenal glands weighing from 10 to 12 gm. per pair were considered normal on gross examination. Twelve pairs ranging from 14 to 26 gm. exhibited evidence of hyperplasia, such as thickening of the cortex and nodularity. The evidence was often equivocal in the smaller glands and clear-cut in the larger ones. The size and shape of the resected segments of gland confirmed the surgeon's estimate of the percentage of adrenal tissue removed. The 4 glands containing adenomas ranged from 20 to 28 gm. each. The control specimens consisted of segments of adrenal glands that measured from 0.5 to 1 cm. in greatest dimension and contained all elements of cortex and medulla. In all instances the cortex was of normal thickness, and no nodularity was seen.

### *Microscopic Observations*

In sections stained with hematoxylin and eosin, the most consistent alteration manifest in all the hyperactive glands was a marked increase in width of the zona fasciculata as compared with that found in the control tissues.<sup>5</sup> In what appeared to be the earliest stage of hyperplasia, the increase in thickness was characterized by an impingement of cells resembling those of the fascicular zone upon the glomerulosa. The latter was frequently narrowed over large areas (Figs. 1 and 2) and occasionally absent; in these circumstances the fascicular elements extended directly to the capsule. In the regions of narrowing, the glomerulosa was occasionally the seat of fibrosis, atrophy of cells, and pyknosis of nuclei; in some glands this occurred focally, in others, diffusely. A distinct transition between the reticular and fascicular zones could not be recognized, and cell cords indistinguishable from fascicular cells extended directly to the medulla. The increase in fascicular tissue and decrease in the glomerulosa resulted in little or no increase of total cortical width, and no increase in gland weight beyond the normal range. In sharp contrast, all the control glands maintained a distinct and well demarcated zona glomerulosa.

In the hyperplastic glands with widening of the fasciculata, irregularity of the cell cords in the outermost portion of this zone and micronodule formation were noted. Nodules were observed in all cases of Cushing's disease but were rare in the glands from control

cases. In the latter, even when present, they were not as well developed as those in the minimally hyperplastic glands.

A characteristic feature which was noted in both the hyperactive and control glands was a zone of cortex that surrounded and was in close apposition to the muscular medullary veins. On cross section this cortical zone itself was often surrounded by medullary tissue. The portion closest to the vein wall resembled zona glomerulosa, and the remainder had the structure of zona fasciculata, a pattern that has been described by other investigators.<sup>6</sup> In the controls the perivenous zone of cortical substance was rarely more than 3 or 4 cell layers in thickness. In the hyperactive gland it was markedly thickened, often nodular, and composed predominantly of cells resembling those of the fasciculata (Figs. 3 and 4). The medulla and the surrounding cortex were frequently compressed and distorted.

Another feature of adrenal cortical stimulation was the presence of large, occasionally huge, cells in the mid and inner zona fasciculata. The cytoplasm was considerably greater in amount than that of normal cells, and was markedly acidophilic and granular. Many of these cells exhibited little cytoplasmic vacuolization; others contained large scattered vacuoles. The nuclei were often strikingly large and hyperchromatic. Mitotic figures were not noted. The cells appeared in scattered foci throughout the cortex in 4 cases of hyperplasia, and in one case formed the predominant cortical element (Figs. 5 and 6). Transitions from normal fascicular cells to atypical cell forms were observed. Although the more atypical cells appeared in clearly hypertrophic glands, transitional forms were frequently present in smaller glands which were of normal weight.

With the Sudan IV stain, a fairly constant quantity of lipid was found in the zona glomerulosa of control glands and in the portions of the glomerulosa in hyperactive glands that were undisturbed by the widened zona fasciculata. Small droplets of lipid were distributed throughout the cytoplasm of most of the cells; it rarely filled the cells. In the areas of glomerular compression, lipid was considerably reduced to absent. The cells of the outer fasciculata in both hyperactive and control glands consistently contained lipid, usually in large conglomerate droplets that fused to fill the cytoplasm and displace the nucleus. In the cortex of the hyperactive glands the uniformity of distribution was often interrupted by nodules. Near the midportion of the fascicular zone, cells containing little or no lipid were more frequent. In the inner cortex (the inner fascicular and the reticular zones) concentrations of lipid were often markedly reduced, and droplets were generally



small enough to appear granular. In general, the transition from lipid-rich to lipid-poor areas tended to be more sharply demarcated and the depletion more complete in the hyperactive glands, where the zone of lipid loss often extended almost to the outer portion of the fasciculata (Figs. 7 and 8). In a few control glands, however, as much lipid was present in the reticular zone as in the outer fascicular region. The concentration of lipid in the reticularis was not seen in any of the hyperactive glands. The reduction of lipid in the cells of the inner fascicular and the reticular zones was largely responsible for the prominent cytoplasmic eosinophilia in this area in sections stained with hematoxylin and eosin. At all levels in the cortex, the cytoplasm of the cells that lacked lipid was acidophilic.

The responses to the Schultze and Windaus procedures are considered together because their distribution was similar. Positive reactive lipids were present in greatest concentration in the outer fascicular zone, paralleling the distribution observed with the Sudan IV staining. In the inner cortical region, however, demonstrable cholesterol showed even greater reduction than was the case with the sudanophilic lipids. This relative decrease was most striking in the hyperactive glands.

Only a small portion of the total lipids reacted in positive fashion to the naphthoic acid hydrazide (NAH) and Schiff reactions, and their distribution did not differ appreciably in control and hyperactive glands. The NAH and Schiff positive lipids tended to concentrate in the outer fascicular zone, appearing as cytoplasmic droplets that occasionally became conglomerate. As might be expected, the distribution of the Schiff and NAH positive lipids was similar, but the intensity of the reaction at a given site often varied considerably; i.e., a weakly positive Schiff and a strong NAH reaction or the reverse.

The zones of cortex surrounding the central veins exhibited the same variation in lipid distribution as the remainder of the cortex. There were some individual differences in the paired adrenals of a given case. With the exception of the glands containing adenomas, however, the pairs were generally quite similar, despite variations in duration of fixation. This was so even in the cases where considerable time had elapsed between operations.

As mentioned above, a characteristic feature of the hyperactive adrenal was the development of nodules in the cortex. These varied considerably in size and definition. Some were obvious to gross inspection, and others were discernible only microscopically. Lipid stains frequently highlighted nodules in glands considered normal or diffusely hyperplastic when stained by routine methods. The evolution



of these structures could be traced in the sections of glands with multiple nodules. The initial alteration, present in all the hyperactive glands, was characterized by a loss of the radial symmetry of the cell cords in the outer fascicular zone so that groups of cells stood out as individual clusters, distinct from the surrounding tissue (Fig. 9). As the nidus of cells enlarged, the process of circumscription continued so that the convexity of the developing nodule formed a concavity in the contiguous cortex (Fig. 10). Sinusoids as well as cellular elements were compressed, although frequently a dilated sinusoid was seen at the periphery. Increase in size of the nodule was sometimes accompanied by condensation of surrounding reticulum. Occasionally, this was followed by collagenization and complete or partial encapsulation. Many large nodules, however, developed without encapsulation. Extension to the surface of the gland permitted the adrenal capsule to serve as part of the capsule. While many of the larger nodules remained homogeneous in cell type, others consisted of fused smaller nodules. In the later stages the nodularity was no longer limited to the outer cortex. Nodules originating in the cortical tissues contiguous to the central vein further distorted the architecture, compressing both medulla and cortex (Fig. 4).

The cells forming the nodule frequently differed from those of the neighboring cortex in lipid content and in distribution of the lipids. A lipid-rich nidus stood out sharply from a surrounding lipid-free cortex, and some nodules were lipid-free in a cortex where fat was abundant (Figs. 9 to 11). In some instances the size of the lipid droplets differed in the nodule and the surrounding cortex. These distinctions could be brought out clearly by the use of the lipid stains. Occasional nodules became obvious because of qualitative differences in lipid content; cells of the nodule might be cholesterol-positive while the surrounding cortex contained lipid but no cholesterol (Figs. 9 to 11). The reverse also occurred. Similar distinctions, dependent on qualitative differences in lipid between intranodular and extranodular cells, could be shown with the other staining methods. In the larger nodules which appeared to result from fusion of several smaller ones, the uniformity of the reaction within the nodule was lost because each of the components frequently retained its own homogeneous staining character. These nodules were usually intracortical, and were composed predominantly of fascicular elements. This was in sharp contrast to a type of nodule that was usually extracapsular, found in both control and hyperactive glands. These contained definite glomerular and fascicular zones with a relationship that duplicated the normal cortical

arrangement. The similarity to the normal cortex was confirmed by the demonstration of a lipid pattern in the nodule identical to that found in the adjacent cortex (Fig. 12). Multiple sections usually revealed a connection composed of a pedicle of cells or fibrous tissue between the extracapsular nodule and the cortex. On the other hand, occasional extracapsular nodules were situated at a distance from the capsule and apparently were not attached. Unencapsulated nests of adrenal cells were often found in the periadrenal fat.

The 4 apparently unilateral, solitary intracortical nodules were of a type generally considered adenomas. They were composed of multiple smaller nodules which appeared to have fused to form the larger mass (Fig. 13). Structural relationships of the cortex were not preserved within the nodule, and it was difficult to assign an origin to the cells. The lipid content varied considerably. Where distinct component nodules could be observed, the cells frequently maintained a qualitative and quantitative uniformity of lipid content, as though they were responding as a unit to whatever stimuli influenced lipid distribution in the tumor.

In 3 of the adenomas no evidence of cellular atypism was noted. In one, large acidophilic cells with large hyperchromatic nuclei, similar to those observed in several cases of hyperplasia, were present throughout the tumor. In two patients with unilateral adenoma a biopsy of the opposite side showed nodularity and predominance of fascicular elements, suggesting a pre-existing hyperplasia. Although there appeared to be some reduction in cortical width, this was not striking (Fig. 14). In two instances the homolateral cortex at a distance from the adenoma showed definite nodular hyperplasia.

#### DISCUSSION

These pathologic observations indicate that the adrenal glands in patients with Cushing's syndrome exhibit histologic and histochemical characteristics differing from those in control glands. In most cases enlargement and nodularity were obvious to gross examination, but in the others histologic examination was necessary to detect evidence of hyperactivity. The extent of these structural alterations could not be correlated with the duration, severity, or any clinical feature of the syndrome. Several patients with severe clinical manifestations showed only minimal histologic lesions.

The use of surgically removed adrenal glands as control material afforded a number of advantages. Post-mortem effects were eliminated, as were the changes which frequently result from prolonged and com-

plicated final illnesses. In addition, the surgical experience could be considered to be a fairly standardized stress, and its effect could be observed in the cortex. The patients were all generally subjected to similar preoperative preparations and similar retroperitoneal procedures.<sup>7</sup> Human variations even under the most ideal conditions raise the question of whether well controlled material is attainable. The factors influencing the status of the adrenal cortex are so diverse that the control glands obtained from cases of sudden death in many studies may well represent a heterogeneous group. Certainly the existence of chronic or even acute disease would, in most instances, make the post-mortem adrenal cortex an excessively stimulated one. Individual emotional reactions to stress may affect the lipid pattern in the cortex rapidly. Even tissue obtained surgically for control purposes leaves much to be desired. Interpretations are made with this in mind.

The narrowing of the glomerular zone observed in the hyperactive glands appeared to be the result of actual compression by expansion of the zona fasciculata. It was not possible to determine whether a portion of the reduction in size of the glomerulosa was the result of the transformation of some of its components into fascicular cells. The ultimate effect, however, was a marked increase in the fasciculata at the expense of the other zones. The relationship between fascicular and glomerular zones appeared to be a dynamic one. It is probable that excessive stimulation of the fasciculata will result in a preponderance of this zone. On cessation or temporary suspension of this stimulation, a more normal relationship may be re-established. Factors which result in stimulation of the glomerulosa cause the converse of this sequence. The histologic pattern of the cortex reflects the sum of these effects in the period before death or surgery.

Compression of the medulla by proliferating cortical elements was a prominent and early feature of cortical hyperplasia. As a result, the medulla failed to appear on many serial sections, and when it was present, it was often distorted by impinging cortical cells. In the control glands, the medulla was much more evident in cross sections and generally occupied a considerably larger area. Thus, while the normal medulla is discontinuous or focally attenuated, cortical hyperplasia results in relative and probably absolute reduction of its size.

Hypertrophied acidophilic cells with nuclear atypism were present in their most striking form in only a few cases. Lesser degrees of the change were more frequent. These cells appeared similar to the large acidophilic elements described in the adrenal cortex of mice overstimulated by an ACTH-producing pituitary tumor.<sup>8</sup> They also re-

sembled the cells occasionally observed in regenerating foci in diffuse cortical atrophy in human beings<sup>9</sup> and bore a striking resemblance to the large acidophilic cells in the corpus luteum. The significance of these similarities must be interpreted with caution. However, the coincidence of this cell type and other signs of hyperplasia, and its absence in control specimens, leads us to believe that the cell form is an indication of unusual stimulation of the adrenal cortex.<sup>10</sup>

Evidence from the study of experimental animals which have undergone stress suggests that depletion of lipids, particularly cholesterol, in the non-atrophic adrenal cortex is an indication of stimulation.<sup>11-13</sup> Depletion of cortical lipid in the course of acute disease has been noted by many investigators,<sup>6,14,15</sup> and marked lipid depletion has been found in patients who have died following the administration of ACTH for varying periods of time.<sup>16</sup>

The pattern of lipid distribution in both the hyperactive and the control glands was certainly influenced by operative maneuvers. The depletion of lipid was greater and the transition from lipid-abundant to lipid-poor zones was more abrupt in the hyperactive than in the control glands. Cholesterol made up a smaller proportion of the total sudanophilic material in the mid and inner zone than in the outer fascicular zone in both groups of cases, but this relative reduction was most marked in the hyperactive glands. The depletion of lipid, when evident, always affected the inner cortex. An outer layer of the fascicular zone, with a thickness of several cells, regularly retained lipid in all cases. This pattern of depletion suggests that lipid is usually removed initially or most rapidly from the innermost cortical zone, and the loss is manifest only later in the outer portion of the fasciculata. The extent of lipid depletion is probably dependent upon the intensity and duration of the stimulus.

The presence of considerable lipid in the zona reticularis of several glands in the control group, while unusual, does indicate that lipid is occasionally present in high concentrations in this region. It is probable that the usually low concentration of lipid in the reticular zone actually reflects mild or normal cortical activity. This suggests that the zona reticularis may represent one of the most persistently and actively secreting areas, with little opportunity for lipid accumulation. The histologic features which distinguish the cell of the reticularis from that of the fasciculata are predicated largely on differences of functional state. Certainly in the few cases where the reticular zone contained considerable lipid, differences could not be clearly defined. It is apparent that regardless of interpretation of function, the regions

occupied by the fasciculata and the reticularis, where they are definable, react in a definite and related pattern insofar as lipid secretion is concerned. No such correlation could be established between the glomerulosa and the other zones. The lipid pattern of the glomerulosa varied slightly from case to case in both hyperactive and control glands. It appeared to be independent of the changes in the remainder of the cortex except where impingement of fascicular cells caused distortion of the glomerulosa in the hyperactive gland.

The lipid demonstrated by the NAH and Schiff reactions failed to provide information for the recognition of hyperactive gland. The stains did serve, however, to indicate qualitative differences in the lipid content of intranodular and extranodular cells. A considerable portion of the lipid reacting to both these reagents probably represented aldehydic carbonyl groups derived from oxidation of unsaturated fatty acids during standing and fixation.<sup>17</sup> These lipids comprised only a small fraction of the total cortical lipid; the relative amounts were the same in both the normal and the hyperplastic cortex.

In all of the hyperactive glands, cortical nodule formation was prominent. Transitional stages between diffuse and nodular hyperplasia made it unlikely that a fundamental difference existed between these forms. The lipids within the nodules, particularly in the early stages of development, often differed quantitatively and qualitatively from those in the extranodular tissue. Indeed, lipid variations often aided in the detection of the early nodule not otherwise apparent in hematoxylin and eosin stained sections. The variations in lipids may be explained by differing rates of secretion and reaccumulation or by focal alterations in metabolism. Nodules and nodular hyperplasia have been produced in the adrenal cortex of animals by a variety of hormonal stimuli.<sup>18-20</sup> In general, the nodular patterns produced and their histogenesis appeared similar to those observed in the hyperplastic cortex of Cushing's disease. It is reasonable, therefore, to consider that the nodules in the cases of Cushing's disease reflected an irregular response by the cortex to excessive or unusual stimulation. The possibility exists that unusual reactivity of cortical cell groups to normal stimuli may also be a factor in nodule production, as may focal failure to react to stimuli acting on the remaining cortical tissue.

Care must be taken to differentiate between the nodule located in the cortex which fails to duplicate the normal cortical pattern and the nodule in extracortical or intracapsular location, often representing an adrenal cortex in miniature. The latter is quite a common occurrence in infants<sup>21</sup> and is also frequently encountered in the normal adult.



These nodules differ from the intracortical nodule and the adenoma in both development and significance. They represent ectopic tissue, or extensions of adrenal tissue beyond the capsule. They become more obvious in conditions associated with adrenal hyperplasia because they respond to the same stimuli as the normally located cortex. Otherwise their mere existence does not indicate hyperplasia or unusual stimulation.

The relationship, if one exists, between the intracortical micro-nodules in hyperplasia and the adenoma is difficult to assess. Histologic and histochemical methods offer only circumstantial evidence suggesting a relationship. Our investigation has shown no sharp distinction between the two conditions. In the specimens containing the adenomas, nodularity and other microscopic features suggestive of pre-existing hyperplasia were observed in the cortex of both the homolateral and contralateral glands. Nuclear atypism of a fairly marked degree was found in adenomas as well as in hyperplastic glands. Histologically, the similarities between the hyperplastic nodule and the adenoma were often striking. However, none of these observations preclude the possibility that the two lesions may have distinctly different pathogenetic pathways. It is probable, however, that some cortical adenomas are initiated in generalized cortical hyperplasia. A single focus in the hyperplastic cortex may develop a potential for growth and, in some instances, for hormone secretion independent of the usual controlling factors. The secretion of this independent nodule is responsible for involution of the remaining cortical tissue and ultimately perhaps for permanent suppression of the original stimulus to hyperplasia. If this hypothesis is valid, it might account for the relatively good clinical results following resection of adenomas.

In the absence of histochemical techniques which specifically identify the biologically active steroids and of practicable methods indicating turnover rates in the cortex, there are no morphologic criteria that necessarily reflect the secretion of these adrenal cortical hormones. It is probable that situations exist in the adrenal analogous to goiter caused by iodine deficiency or thiouracil, from which, despite hyperplasia, little effective hormone is secreted. In some instances, the adrenal alterations may reflect the results of a stimulus no longer in action. It will be noted that the histologic features of hyperplasia in surgical specimens we have described are common in some degree in many, perhaps the majority, of adrenals examined at necropsy. Cortical hyperplasia has been reported at necropsy in human adrenal glands in association with many diseases not primarily of endocrine nature. A

study of post-mortem specimens in progress in our laboratory has shown an association of marked adrenal cortical hyperplasia in adults with prolonged chronic systemic disease. Indeed, unless specimens are carefully selected, the adrenal gland at necropsy is in some degree hyperplastic when compared with surgical controls. Thus the histologic criteria for the normal adrenal based on unselected necropsy material may be misleading. A variety of stimuli of differing origins may initiate a sequence of events leading to these adrenal cortical alterations via a final common path. Where the adrenal response is proportionate to the demand made upon the gland, the syndrome of hyperadrenocorticism is not induced; yet the microscopic structure of the cortex may reflect unusual stimulation. Where the cortical response is initiated spontaneously or is disproportionately great for the demand which provokes it, hyperadrenocorticism in some degree may supervene. The morphologic alterations in the adrenal cortex in Cushing's disease may differ neither qualitatively nor quantitatively from those accompanying ordinary physiologic response to stress. It is apparent that morphologic observations must be considered in conjunction with clinical and laboratory data before final interpretations can be made.

#### SUMMARY

Specimens of adrenal cortex removed surgically from patients with hyperadrenocorticism of the Cushing type have been subjected to conventional staining and to histochemical techniques for the demonstration of lipids. As controls, biopsy specimens were procured from normal adrenal glands during retroperitoneal surgical procedures in patients without evidence of endocrine disease, hypertension, or debilitation.

The development of hyperplasia was traced from apparently early stages to well developed nodulation. Characteristics were described which may lead to more complete understanding of the pathogenesis of cortical hyperplasia. In its initial phase the process was characterized by increased thickness of the zona fasciculata at the expense of the glomerulosa and medullary region. Initially this occurred without increase in total gland weight above normal levels. Lipid distribution was similar to that of the normal control, but depletion of sudanophilic material and particularly cholesterol was more marked. Cellular atypism noted in a number of the hyperplastic glands was considered an indication of unusual stimulation.

A comparison of adenomas and the nodulations noted in hyperplastic glands indicated that in at least some cases, nodular hyperplasia preceded the appearance of a cortical adenoma.



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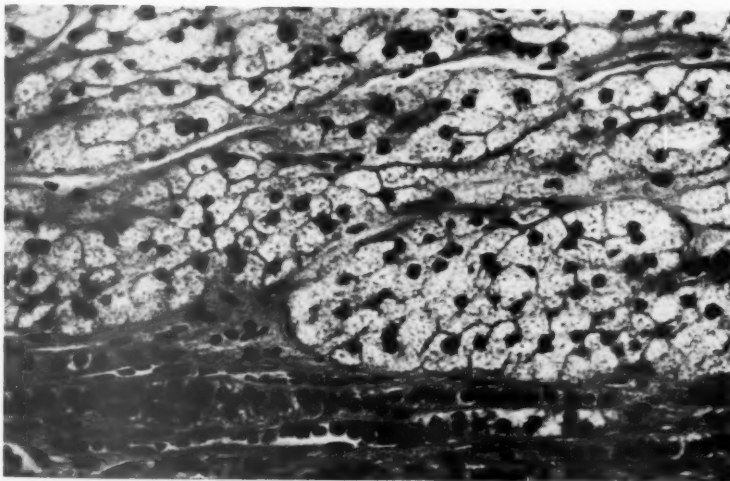
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#### LEGENDS FOR FIGURES

- FIG. 1. Hyperplastic cortex with compression and distortion of the zona glomerulosa by impingement of the zona fasciculata. Hematoxylin and eosin stain.  $\times 120$ .
- FIG. 2. Hyperplastic cortex showing the relation of the zona glomerulosa to the zona fasciculata. Hematoxylin and eosin stain.  $\times 215$ .



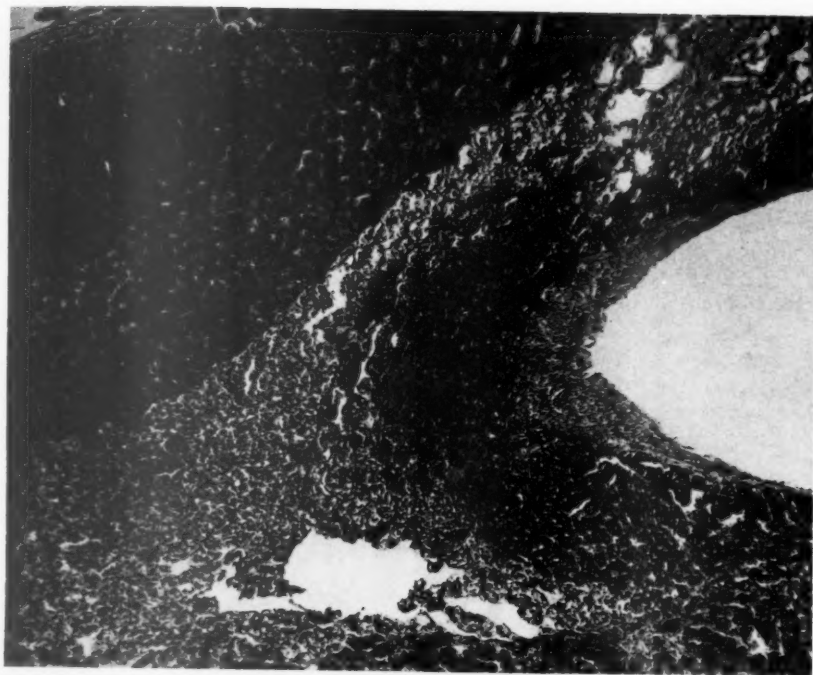
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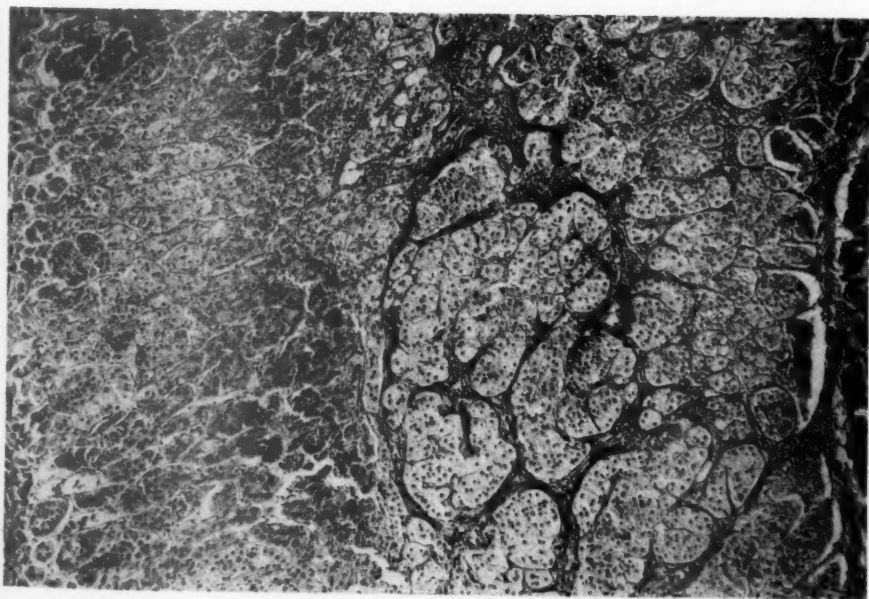
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FIG. 3. Hyperplastic cortex with a lipid filled zone of cortical cells closely apposed to the muscular central vein. In this field the degree of hyperplasia is only slightly greater than that found in several of the control glands. Sudan IV stain, frozen section.  $\times 45$ .

FIG. 4. On the far right is a portion of the muscular coat of the central vein. On the left is the cortex. The central portion of the field is occupied by a nodular proliferation of cortical cells which compress and distort the medulla. Hematoxylin and eosin stain.  $\times 80$ .



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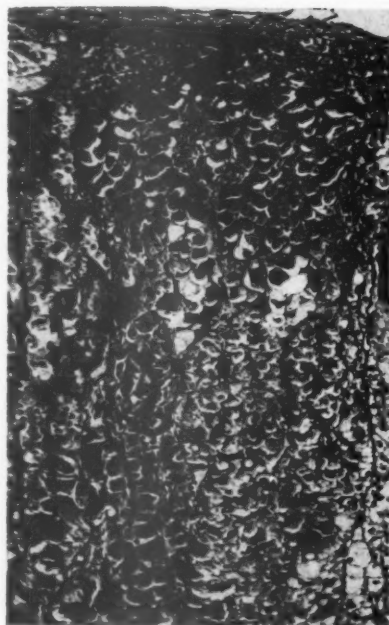
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FIG. 5. A portion of a hyperplastic cortex occupied by large, lipid-poor cells with abundant granular, acidophilic cytoplasm and often with large, hyperchromatic nuclei. Note the compression of the zona glomerulosa. Hematoxylin and eosin stain.  $\times 150$ .

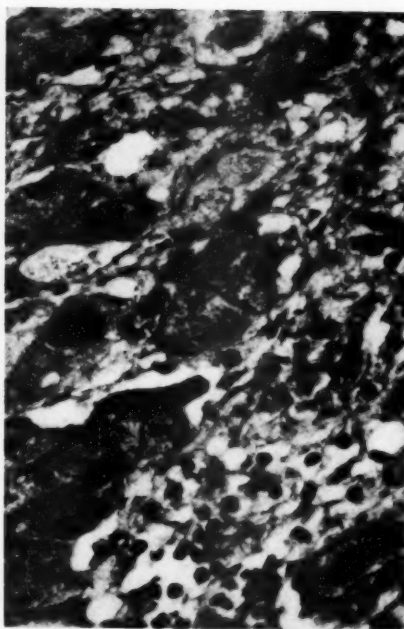
FIG. 6. A higher power view of the field shown in Figure 5. Hematoxylin and eosin stain.  $\times 430$ .

FIG. 7. Marked depletion of lipid in the inner zone of the cortex in a hyperplastic gland. Sudan IV stain, frozen section.  $\times 86$ .

FIG. 8. Normal adrenal. Lipid depletion is of considerably less degree than that encountered in cortical hyperplasia. Sudan IV stain, frozen section.  $\times 90$ .



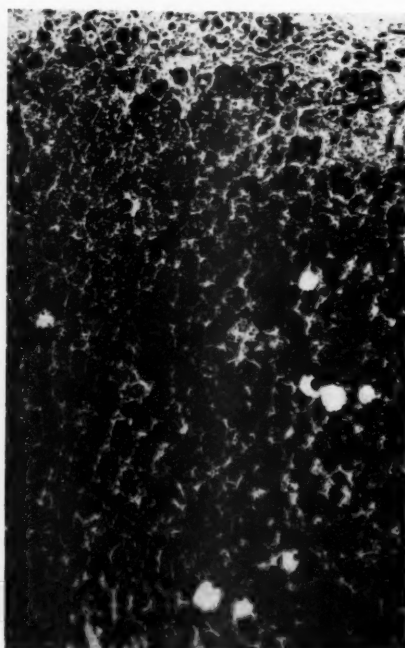
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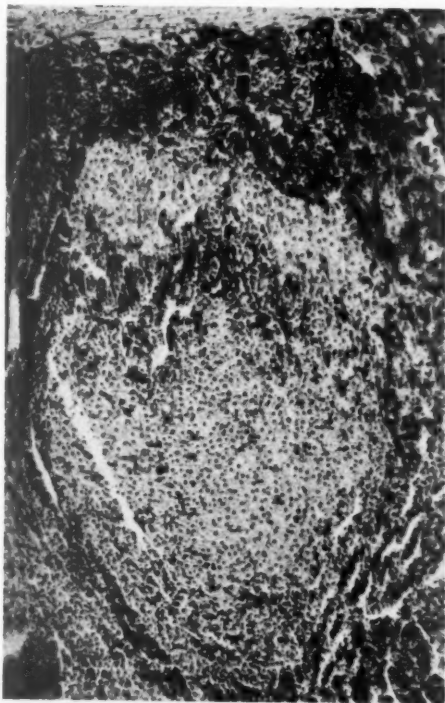


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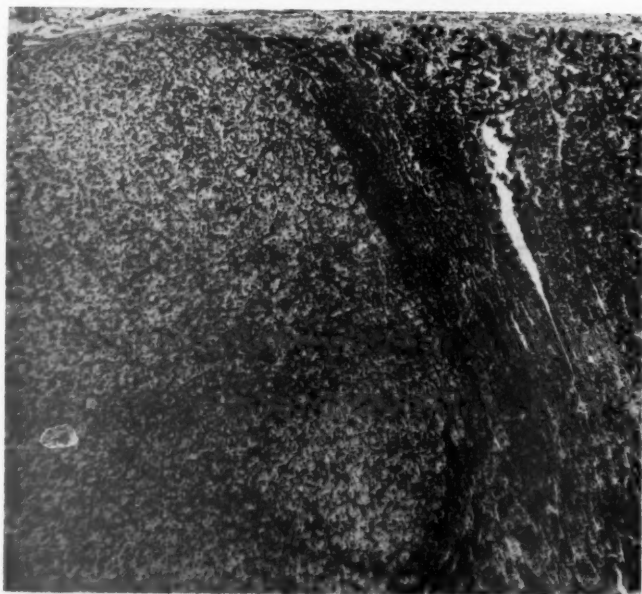


FIG. 9. An early nodule in the outer zona fasciculata of a hyperplastic gland. It stands out in sections stained for lipids because of the predominance of lipid-free cells in the nodule. It was not sharply defined in hematoxylin and eosin stained sections, where it was characterized by loss of radial symmetry, disorganization of fascicular cell cords, and slight compression of the surrounding cortex. Sudan IV stain, frozen section.  $\times 120$ .

FIG. 10. A well developed nodule compressing the surrounding cortex and extending to the capsule. The nodule is sharply defined in this section because the abundant lipid in its cells is predominantly Schiff-stain negative. The nodule was much less sharply defined in hematoxylin and eosin and Sudan stained sections since the intranodular and extranodular lipid reacted in similar manner. Schiff stain, frozen section.  $\times 80$ .



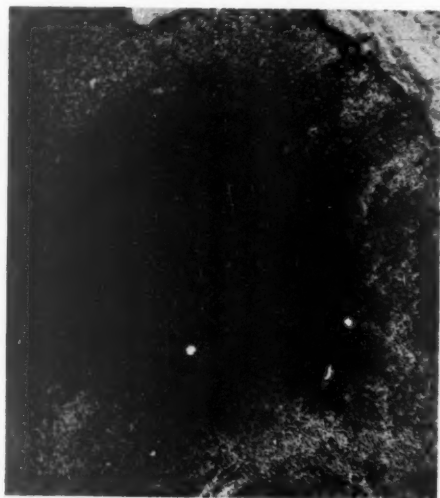
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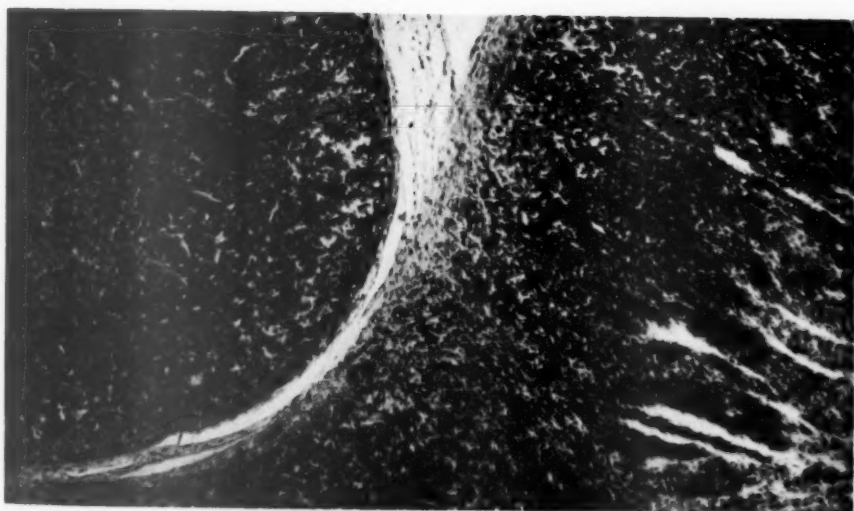
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FIG. 11. A sharply defined nodule in a hyperplastic cortex characterized by an abundance of Schultze-positive lipid. The surrounding cortex contained considerable sudanophilic material but was comparatively poor in lipids reacting to the Schultze procedure. Schultze stain, frozen section.  $\times 100$ .

FIG. 12. An extracapsular nodule in a normal adrenal. In this instance the nodule clearly represents an extension of the cortex. Zonation and lipid pattern are identical in the cortex and the nodule. Sudan IV stain, frozen section.  $\times 60$ .



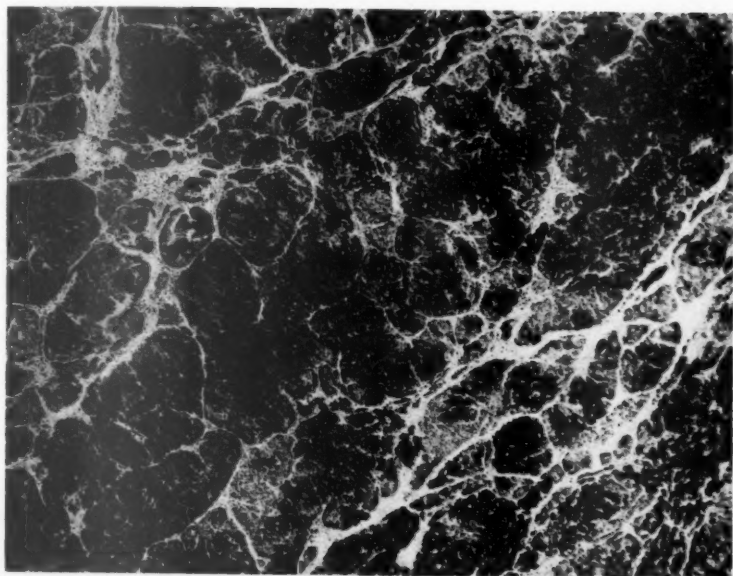
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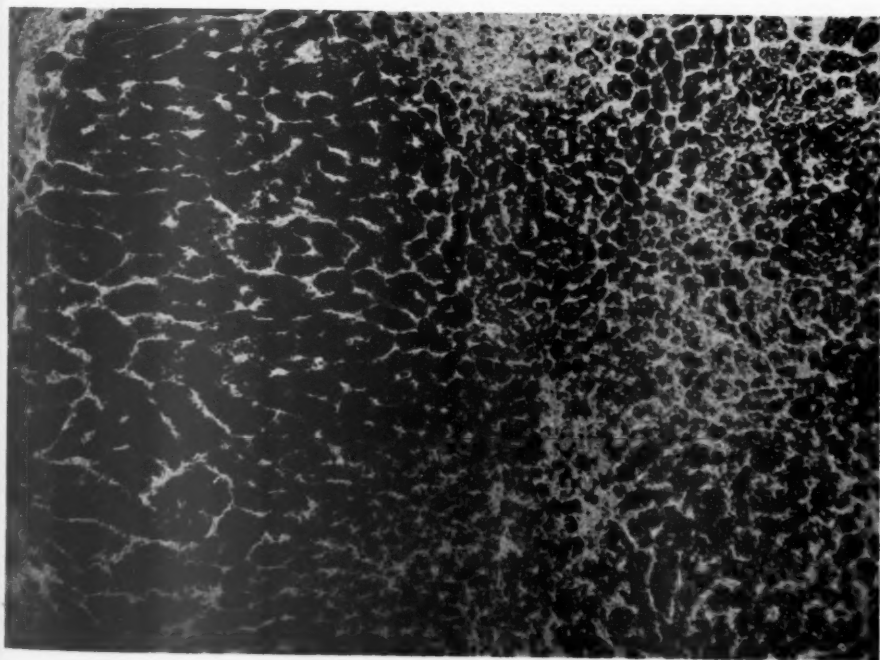
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FIG. 13. A section demonstrating the multinodular character observed in some adenomas. The nodules composing the tumor showed variations in lipid pattern as in the case of hyperplastic nodulation. Sudan IV stain, frozen section.  $\times 32$ .

FIG. 14. Cortex of the contralateral adrenal in the patient with the adenoma shown in Figure 13. Despite the activity of the adenoma, the cortical width appears only slightly reduced. Zona fasciculata cells predominate, and a tendency toward nodularity is evident. Sudan stain, frozen section.  $\times 120$ .



13



14





THE RELEASE OF ACID PHOSPHATASE AND BETA-GLUCURONIDASE  
FROM CYTOPLASMIC GRANULES IN THE EARLY  
COURSE OF AUTOLYSIS\*

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Among pathologic conditions, necrosis is frequently encountered either isolated or in association with inflammation, infection, or tumors. Despite the frequency and importance of necrosis in pathologic lesions, little is known of the early biochemical changes occurring in this process. One of the difficulties in approaching the problem is that necrosis is seldom found as an isolated feature *in vivo* since humoral and cellular reactions are also induced in the organism. Hence, it seems preferable to study these early biochemical mechanisms in a similar condition which can be controlled *in vitro*, namely, autolysis.

In the present study, the changes in the intracellular distribution of enzymes in the early course of autolysis were investigated because it was felt that they might be released into the cellular supernatant and so play an important role in the outset of the autolytic process. The hydrolytic enzymes are prominent among those which are likely to play a significant role in cellular digestion. In relation to the intracellular distribution of this group of enzymes, it is interesting that most of them are present in high concentration in a specific granule, the lysosome.<sup>1</sup> Although these organelles have not been identified morphologically, they have been well defined by their content of enzymes and sedimentation characteristics in 0.25 M sucrose.<sup>1,2</sup> Methods allowing the determination of the amount of free and bound enzymes in liver homogenates were developed, and it was found that 30 per cent of them were free while 70 per cent were bound to cytoplasmic granules.<sup>3</sup> Among the numerous enzymes found in these granules, large amounts of acid phosphatase and beta-glucuronidase were constantly present; therefore, these compounds may be considered as excellent reference enzymes for these granules. This study was concerned essentially with the determination of free and bound acid phosphatase and beta-glucuronidase in mouse liver after 30 minutes to 2 hours of autolysis. The data on the hydrolytic enzymes was also correlated with alterations in nitrogen, deoxyribonucleic acid (DNA)

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and ribonucleic acid (RNA), cytochrome oxidase and glucose-6-phosphatase during autolysis. The latter enzymes were selected because 80 per cent of their activity is bound to the mitochondria in the case of cytochrome oxidase, and to the microsomes in the case of glucose-6-phosphatase. Consequently, the variations of their activity in the course of autolysis might reflect the fate of those organelles.

#### MATERIAL AND METHODS

##### *Animal and Tissue Preparation*

Mice were used in these experiments to allow comparison with observations previously recorded in the literature concerning autolysis.<sup>4</sup> Six-months-old albino mice were starved for 12 hours before sacrifice. The animals were beheaded and thoroughly bled. The liver was excised immediately after sacrifice, and the gall bladder was separated from the liver. The control livers were immediately placed in ice cold 0.25 M sucrose, and homogenized in the same medium. The others were autolyzed for 30, 60, and 120 minutes by the method described by Berenbom, Chang, Betz and Stowell.<sup>4</sup>

At the end of the period of autolysis, the specimens were chilled in 0.25 M sucrose and homogenized. The tissue was regularly homogenized for 2 minutes with a commercial model of the Potter-Elvehjem homogenizer equipped with a Teflon pestle. During this procedure, the temperature of the tissue was kept near 0° C. by maintaining the homogenizer in cracked ice. The enzymatic assays were performed immediately after homogenization, and samples were stored in the freezer at -20° C. for chemical analysis.

Free and total acid phosphatase and beta-glucuronidase activities were determined as described by Wattiaux and de Duve<sup>3</sup> except that the preparation of the homogenate for total beta-glucuronidase determination was slightly modified. Instead of carrying out the determination in the presence of Triton X-100, the homogenate was blended for 3 minutes in a Waring blender, cooled by means of a plastic jacket in which ice cold water was circulated. Triton X-100 was avoided in the total beta-glucuronidase determination because after autolysis it would prevent complete protein precipitation and lead to high blank values. Cytochrome oxidase and glucose-6-phosphatase were assayed as described previously.<sup>5</sup>

Phenolphthalein beta-glucuronide was obtained from the Sigma Chemical Company and recrystallized as described by Talalay, Fishman and Huggins.<sup>6</sup> Sodium beta glycerophosphate was obtained from Eastman Kodak Company, sodium glucose-6-phosphate and cytochrome C were purchased from the Sigma Chemical Company and

utilized without further purification. The DNA standard was prepared with Worthington DNA dialyzed against 0.1 M acetic buffer (pH 5). The preparation contained 0.09 mg. of phosphorus per gm. of DNA. The RNA standard was yeast RNA purified by the method of Vischer and Chargaff.<sup>7</sup> It contained 0.78 mg. of phosphorus per mg. of RNA.

#### *Analytic Methods*

The nitrogen was determined by the Kjeldahl distillation method on a sulfuric acid digest. The DNA and RNA were determined by the diphenylamine<sup>8</sup> and the orcinol<sup>9</sup> methods on the hot trichloroacetic extract obtained by the method of Schneider.<sup>10</sup> The acid soluble phosphorus was determined by the Fiske and Subbarow method.<sup>11</sup>

No changes were found in the amount of nitrogen per gram of wet weight after 2 hours of autolysis in 3 separate experiments. To speed up the experimental procedure, the tissues were homogenized and brought to volume without weighing the original liver fragments. Hence, except for the 2 experiments where the nitrogen values are given in milligrams per gram of wet weight, the data are expressed per milligrams of nitrogen, the latter being determined on a sample of the homogenate. The acid phosphatase, beta-glucuronidase and glucose-6-phosphatase activities are presented in micromoles of substrate digested after 10 minutes of incubation, in the conditions of the assay of an amount of homogenate corresponding to 10 mg. of nitrogen. Cytochrome oxidase is expressed in Cooperstein and Lazarow units<sup>12</sup> present in a quantity of homogenate corresponding to 10 mg. of nitrogen.

#### RESULTS

The changes in total free and bound acid phosphatase, and beta-glucuronidase in the control liver fragments and in the fragments obtained 30, 60, and 120 minutes after autolysis are shown in Table I. It appeared that the free enzyme activities increased very rapidly while no significant changes in total enzyme activities were observed. The free acid phosphatase activity, which was 42 per cent of the total activity in the control, reached 82 per cent after two hours of autolysis. The bound enzyme activity decreased as shown in Text-figure 1. Table I and Text-figure 2 demonstrate that the changes in total free and bound beta-glucuronidase paralleled those of acid phosphatase. However, the amounts of free acid phosphatase exceeded the amount of free beta-glucuronidase significantly in the controls and in the fragments obtained after 30 and 60 minutes of autolysis.

It appears from Table II that no significant loss in DNA, RNA, and nitrogen was detected after 2 hours of autolysis. However, at that

TABLE I  
*Effect of Autolysis on Acid Phosphatase and Beta-Glucuronidase Activity*

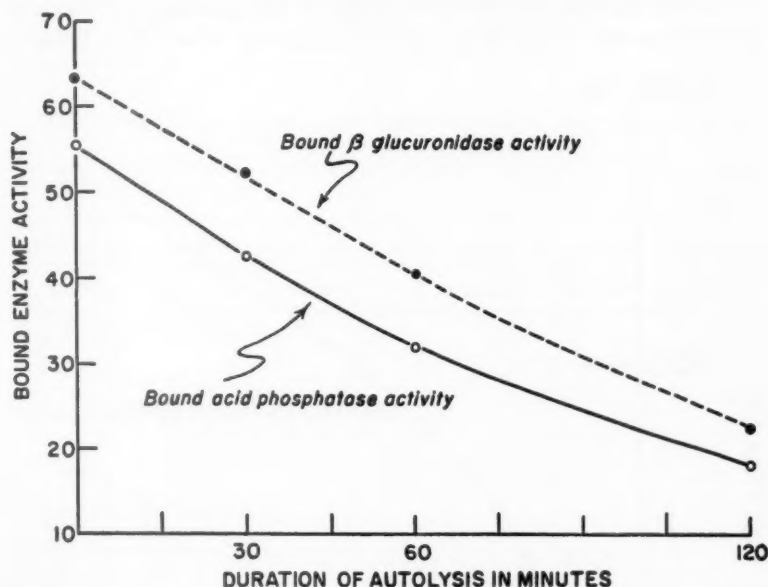
| No. of animals used | Duration of autolysis (min.) | Acid phosphatase activity |           | Beta-glucuronidase activity |            |               |
|---------------------|------------------------------|---------------------------|-----------|-----------------------------|------------|---------------|
|                     |                              | Total                     | Free      | Per cent free               | Total      | Free          |
| 6                   | 0                            | 9.05±0.94                 | 4.05±0.60 | 44.8±3.5                    | 0.66±0.012 | 0.24±0.05     |
| 3                   | 30                           | 9.86±0.89                 | 5.70±0.79 | 57.8±3.64                   | 0.60±0.04  | 0.28±0.02     |
| 5                   | 60                           | 9.12±0.93                 | 6.18±0.59 | 67.8±5.7                    | 0.62±0.05  | 0.37±0.03     |
| 4                   | 120                          | 7.84±0.14                 | 6.40±1.03 | 81.6±6.34                   | 0.55±0.02  | 0.43±0.01     |
| <i>p</i>            |                              | >1                        |           |                             | >2.5       |               |
|                     |                              |                           |           |                             |            | Per cent free |
|                     |                              |                           |           |                             |            | 36.4±2.8      |
|                     |                              |                           |           |                             |            | 46.7±4.1      |
|                     |                              |                           |           |                             |            | 59.7±1.1      |
|                     |                              |                           |           |                             |            | 78.2±4.4      |
|                     |                              |                           |           |                             |            | <i>p</i>      |
|                     |                              |                           |           |                             |            | <0.1          |
|                     |                              |                           |           |                             |            | <0.5          |
|                     |                              |                           |           |                             |            | <0.5          |
|                     |                              |                           |           |                             |            | >1            |

The data give the mean of the individual determination  $\pm$  the standard deviation of the mean. The percentage of free enzyme activity was calculated by taking the total enzyme activity of each individual experiment as 100 per cent. The values were determined by applying the *t* test to the means. The *t* values were calculated either to test the significance of the differences between the amounts of free acid phosphatase and free beta-glucuronidase or to evaluate the significance of the changes in total enzyme activities occurring after two hours of autolysis.

In the first case (*p* values of the vertical column), the mean percentage of free activity of acid phosphatase was compared to the percentage of free activity of beta-glucuronidase. This was carried out in fresh tissues and in livers obtained after 30, 60, and 120 minutes of autolysis. In the second case (*p* values of the horizontal column), the mean of the total activity of acid phosphatase or beta-glucuronidase in fresh tissue was compared to the mean of the total activity of the homologous enzyme after two hours of autolysis.

time there was a slight drop of the cytochrome oxidase and the glucose-6-phosphatase activity (up to 20 per cent) and a considerable increase in acid soluble phosphorus. The latter alteration is better demonstrated in Text-figure 2 where the amount of inorganic phos-

#### CHANGE IN BOUND ENZYME ACTIVITY DURING AUTOLYSIS



Text-figure 1. The mean of the percentage of bound hydrolase (in ordinate) is plotted against the duration of autolysis in minutes. The bound hydrolase activity was calculated by subtracting the free activity from the total enzyme activity in each liver fragment. The bound activity was then expressed as a percentage of the total activity. The mean plotted here is that of the individual percentages.

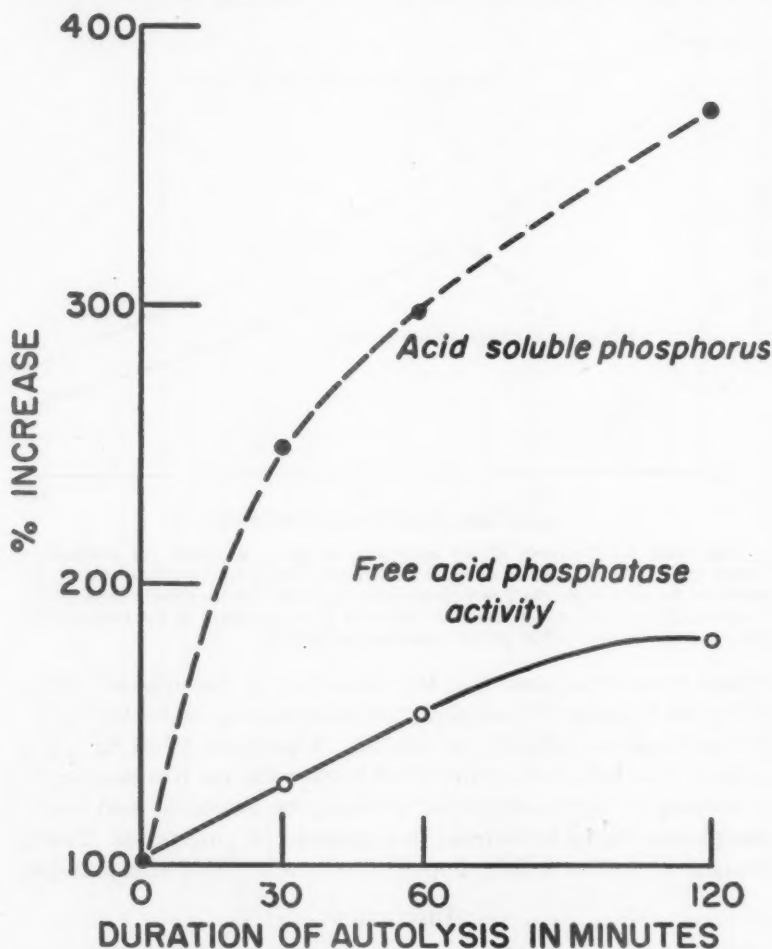
phorus released is plotted as the percentage of the original values. When the logarithm of the percentage of increase in acid soluble phosphorus is plotted against the duration of autolysis (Text-fig. 3), it appears from both Text-figures 2 and 3 that while the free enzyme had a tendency to increase as an arithmetical progression, the acid soluble phosphorus tended to increase in a geometrical progression. Finally the table shows that a slight drop of pH occurred 2 hours after autolysis.

#### DISCUSSION

The observations in this study raise several questions: namely, the reasons for the constant differences in the amounts of free beta-glucuronidase and acid phosphatase; the mechanism of the release of these

enzymes during autolysis; and the effect of the released enzymes on the cell.

It is interesting that in contrast with the observations on rat liver,<sup>1,2</sup> the free acid phosphatase activities in mouse liver were consistently higher than those of the free beta-glucuronidase. The interpretation of this should, of course, take into account the intracellular distribution of the two enzymes and the permeability of the granules. At least two different spatial distributions can be conceived; either there exists a

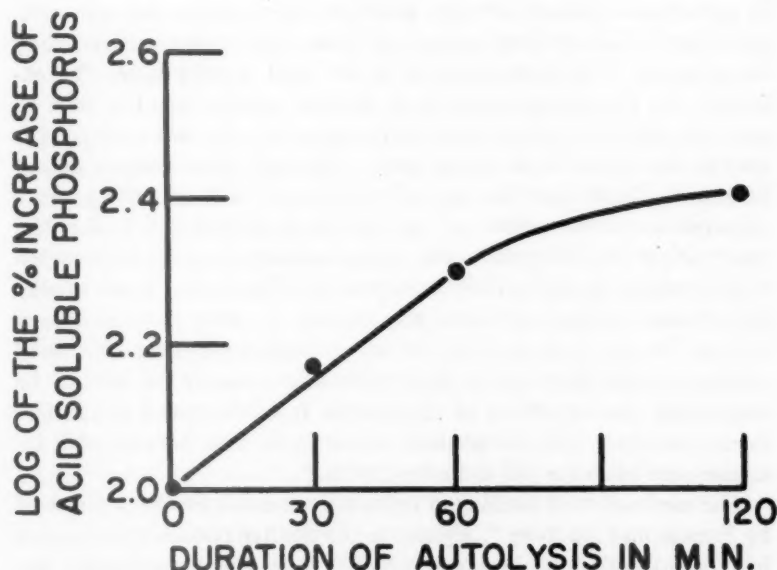


Text-figure 2. The increase of acid soluble phosphorus and of free acid phosphatase is plotted as a percentage of the values for these two compounds at times 0 (= 100%) versus the duration of autolysis.

TABLE II  
Effect of Autolysis on Cell Content

| Duration of autolysis (min.) | $\mu\text{g. DNA/mg. of tissue N}$ | $\mu\text{g. RNA/mg. of tissue N}$ | $\mu\text{g. phosphorus/mg. of tissue N}$ | $\text{mg. N/mg. of wet wt.}$ | Cytochrome oxidase activity | pH                     |
|------------------------------|------------------------------------|------------------------------------|---|-------------------------------|-----------------------------|------------------------|
| 0                            | $156 \pm 23$<br>(5)                | $316 \pm 33$<br>(6)                | $126 \pm 5$<br>(3)                        | $34 \pm 0.84$<br>(3)          | $14.5 \pm 0.3$<br>(4)       | $6.9 \pm 0.02$<br>(3)  |
| 30                           | $182 \pm 25$<br>(3)                | $313 \pm 26$<br>(3)                | $324 \pm 26$<br>(4)                       |                               |                             |                        |
| 60                           | $166 \pm 21$<br>(4)                | $341 \pm 28$<br>(5)                | $374 \pm 24$<br>(4)                       |                               |                             |                        |
| 120                          | $124 \pm 16$<br>(3)                | $336 \pm 52$<br>(3)                | $469 \pm 14.6$<br>(3)                     | $34.2 \pm 1.5$<br>(3)         | $10.6 \pm 2.3$<br>(4)       | $6.56 \pm 0.02$<br>(3) |
| p                            | >0.5                               | >5                                 |   |                               | 0.5                         | <0.1                   |

The table gives the mean of the individual determinations  $\pm$  the standard deviation; the *t* values were determined as for the total acid phosphatase activity, to test the significance of the changes occurring after two hours of autolysis for DNA, RNA, cytochrome oxidase activity and inorganic phosphorus. The number in parentheses refers to the number of individual determinations.



Text-figure 3. The log of the percentage increase in acid soluble phosphorus (value at time X — value of time 0 = 100%) is plotted against the duration of autolysis.



specific granule for acid phosphatase and another for beta-glucuronidase, or the two enzymes are contained in the same granule. Although the methods used here do not discriminate between these two possibilities, in either case this study might shed some light on the permeability of the granule for both enzymes under investigation. To explain the presence of free acid phosphatase in excess of the free beta-glucuronidase, it could be assumed that the granules are more permeable to the former enzyme than to the latter. However, it should then also be expected that the acid phosphatase would be released faster than the beta-glucuronidase during autolysis. As it appears in Text-figure 1, this is not the case. In the absence of an increased permeability for acid phosphatase, it is improbable that the excess free enzyme originates from the granules containing the bulk of it. Hence, at least part of the free enzyme in the fresh homogenate is probably different from the bound enzyme. This view is supported by some recent observations to be published in more detail later. These have indicated that the free acid phosphatase is more thermolabile than the bound enzyme and that the bound enzyme is more easily inhibited by sodium fluoride than the free enzyme.

From their investigation of autolysis, Berenbom and co-workers<sup>4</sup> concluded that the first biochemical change which occurred was a drop in cytochrome oxidase activity. However, they studied this phenomenon after relatively long periods of time, and they restricted their investigation of acid phosphatase to the total activity only. We observed that the drop of cytochrome oxidase activity was less than 20 per cent whereas the increase of free enzyme activity was over 100 per cent of the values in the fresh livers. Although these findings do not necessarily imply that the drop of cytochrome oxidase activity was a consequence of the release of the hydrolytic enzymes, it is doubtful that a slight drop in cytochrome oxidase activity would induce such a drastic release of the hydrolytic enzyme in a free form. Much smaller cytochrome oxidase activities are present in other normal tissues without having such an effect on the granule membrane. The small changes in pH observed in these experiments cannot be held to be responsible for the release of the enzyme from the bound to the free form since free acid phosphatase activities in liver homogenates increase only when the pH drops to 5 units.<sup>13</sup>

The mechanism of swelling of aging mitochondria has been reviewed by Ernster and Lindberg.<sup>14</sup> Alteration in oxidative phosphorylation and loss of adenosine triphosphate (ATP) from the mitochondria are suspected to play an important role in this process. An analogous

mechanism might well be responsible for the swelling and consecutive rupture of the lysosomes. Changes in oxidative phosphorylation and the release of ATP during autolysis are presently under investigation in this laboratory.

While in normal liver the substrate is separated from the enzyme by the normal compartmentation of cells, during autolysis the enzymes are released from their granules. This brings the substrate and the enzymes into closer contact, leading to hydrolysis of the substrate. This explains the increase of inorganic phosphorus which accompanies release of acid phosphatase. The fact that the increase of inorganic phosphorus is logarithmic while the increase of free acid phosphatase is arithmetic suggests that the release of a relatively small amount of enzyme might be very damaging to the biochemical integrity of the cell. This, however, cannot be considered as a general rule. De Duve and his colleagues<sup>2</sup> showed that deoxyribonuclease and ribonuclease can be expected to be released at the same rate as acid phosphatase, yet no loss of DNA and RNA was observed after two hours of autolysis. The release of these enzymes and their effects on the intracellular substrate are presently under investigation. In any case, this study clearly demonstrates that marked changes occur in the intracellular activity of some hydrolases, leading to chain reactions deleterious to the cell. It also appears that in investigating the *primum movens* of autolysis, one should be concerned mainly with those alterations occurring within 30 minutes after the onset of the process.

The reasons for investigating autolysis in preference to necrosis have been indicated at the outset. In view of the similarities between the two entities it is probable that changes analogous to those encountered in the course of our studies may occur in at least some forms of necrosis. Indeed, an increase of free deoxyribonuclease has been described following the administration of x-radiation.<sup>15</sup> The determination of the degree of activity of the hydrolases in free and bound states may have great value in the early detection and the quantitation of these retrogressive states.

The present investigation not only demonstrates some interesting features in the mechanism of autolysis or necrosis; it also illustrates a basic principle pertinent to the interpretation of enzymatic alterations in biology in general. The considerable changes in free enzyme activity, occurring in this experiment in the absence of alterations of total activity, indicate that in the study of pathologic conditions, the determination of total enzyme activities may be of little value. This certainly would be the case if no effort was made to evaluate also the

true enzyme activity (in this instance, free enzyme activity) in the normal cell and its variations under experimental conditions. Moreover, since in the normal cell only small amounts of some enzymes are active, in determining total enzyme activity, one should be certain that the conditions of the experiment (in the present instance, the addition of Triton X-100) permit the measurement of all potential activity present in the homogenate.

#### SUMMARY

The release of acid phosphatase and beta-glucuronidase from their cytoplasmic granules was studied after autolysis of mouse liver for 30, 60, and 120 minutes. These changes were compared with the alteration in total activity of acid phosphatase and beta-glucuronidase and with the activities of cytochrome oxidase, glucose-6-phosphatase, and the content of DNA, RNA, and nitrogen in the liver. The bound acid phosphatase and beta-glucuronidase were rapidly released from their granules in the course of autolysis, while no changes were observed in total acid phosphatase or beta-glucuronidase activities. After two hours of autolysis, only a 20 per cent drop in cytochrome oxidase and glucose-6-phosphatase was observed, while DNA, RNA, and nitrogen values remained unchanged.

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## CELL DEATH

### II. THE EFFECT OF INJURY ON THE ENZYMATIC PROTEIN OF EHRLICH TUMOR CELLS \*

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In an earlier paper it was shown that injured Ehrlich ascites tumor cells exhibited an immediate drop in the mitotic index, accompanied by a loss of nucleic acid and protein.<sup>1</sup> However, in this early stage of damage, the cells showed little change in structure when examined by phase microscopy. The cells refused the entrance of a vital dye into the nucleus and often when fixed and stained with hematoxylin and eosin, could not be distinguished from cells in the original control sample. Of necessity, alterations in physiologic function must eventually result in changes in structure. However, the light microscope does not have the resolution to determine early alterations in the endoplasmic reticulum, and electron microscopists have difficulties in this regard because of lack of knowledge of normal structure.

There has been much discussion regarding the large amount of irradiation necessary to destroy proteins in a pure solution and the smaller amount which is apparently able to inactivate substances *in vivo*. One reason advanced for this discrepancy is that only a slight alteration is necessary to inhibit biosynthetic processes as distinguished from actual destruction of substances already formed within the cell.

The various physiologic processes evaluated in this investigation unfortunately do not measure biosynthetic processes directly. They are concerned principally with apparent defects in the degradation of carbohydrates. However, all synthetic processes do require energy, and interruption in the glycolytic or respiratory pathways will certainly prevent the formation of adenosine triphosphate. Finally, since many enzymes necessary for carbohydrate alteration may have rapid turnover rates, inhibition of protein synthesis may quickly produce enzymatic deficiencies and the observed deviation in metabolic pathways.

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## EXPERIMENTS

In this group of experiments the Ehrlich tumor cells were handled in a manner identical to that reported in the first paper of this series.<sup>1</sup> In addition, the following procedures were carried out:

*Dehydrogenase Activity.* Thunberg tubes were used, containing 2 cc. of Krebs-Ringer solution (0.01 M glucose added) and 0.5 cc. of N/15 methylene blue, with 0.5 cc. of tumor cell suspension added in the side arm. After the air was removed by means of a vacuum pump, the cells were mixed with the dye, and leukomethylene blue formation was measured by means of a Coleman Junior Spectrophotometer at 540 m $\mu$ .

*Respiration.* Standard Warburg manometers were used. Each 15 cc. Warburg flask contained 3 cc. of cell suspension (total  $15 \times 10^6$  cells). To inhibit bacterial growth, 0.05 cc. of penicillin (100,000 units) was added. In addition 0.3 cc. of 20 per cent sodium hydroxide was placed in the center well. When anaerobic experiments were performed, continuous flushing of purified nitrogen through modified Erlenmeyer flasks provided a continuous anaerobic atmosphere for the cells.

*Lactic Acid.* This was determined by the colorimetric method of Barker and Summerson<sup>2</sup> on both the complete cell suspensions and the extracellular medium after removal of the cells by centrifugation.

*Stains.* (1) Cell suspension (0.5 cc. aliquot) was placed in a one per cent solution of osmic acid and stained for fat with Sudan IV. (2) Cell suspension (0.5 cc. aliquot) was placed in a potassium bichromate solution and stained with aged hematoxylin for mitochondria. All sections were stained in their respective stains as a complete group.

## RESULTS

*The Effect of Injury on Dehydrogenase Activity*

If specific substrates are given to starved Ehrlich tumor cells in an evacuated Thunberg tube containing methylene blue, the amount of dye reduced may be an approximate measure of the amount of a specific dehydrogenase present rather than the total of all dehydrogenase activity. As shown in Table I, the greatest dehydrogenase activity was manifested on incubation with two sugars, glucose and mannose, and two amino acids, phenylalanine and tryptophan. The results are recorded as the amount of nonreduced methylene blue remaining after 10 and 30 minutes.

The total dehydrogenase activity of the cell was used for 4 purposes. The first was to obtain an indication of the endogenous reserves of



the cell. These cells contain unusual amounts of endogenous substrates compared with normal tissues. Text-figure 1 shows that not until 2.3 hours had passed was the addition of exogenous glucose of value in the reduction of methylene blue. It did not appear to matter whether the

TABLE I  
Dehydrogenase Activity of Ehrlich  
Tumor Cells

| Substrate        | Per cent of methylene blue non-reduced |               |
|------------------|--|---------------|
|                  | After 10 min.                          | After 30 min. |
| Glucose          | 64                                     | 1.7           |
| Mannose          | 58                                     | 10            |
| DL-phenylalanine | 74                                     | 17            |
| L-tryptophan     | 79                                     | 20            |
| Glutamic acid    | 70                                     | 20            |
| Serine           | 83                                     | 21            |
| Aspartic acid    | 83                                     | 25            |
| Fructose         | 72                                     | 27            |
| Glycine          | 80                                     | 30            |
| Pyruvic acid     | 81                                     | 33            |
| Malic acid       | 87                                     | 36            |
| Lactic acid      | 88                                     | 40            |
| Succinic acid    | 83                                     | 44            |
| Citric acid      | 83                                     | 45            |
| Isoleucine       | 77                                     | 45            |
| Leucine          | 77                                     | 47            |
| Arabinose        | 81                                     | 48            |
| Valine           | 83                                     | 56            |
| L-methionine     | 81                                     | 66            |
| Threonine        | 82                                     | 70            |
| Malonic acid     | 84                                     | 71            |
| Galactose        | 79                                     | 78            |

glucose was added initially or given at the end of this period; it was utilized equally well in the experiments carried out 4.2 hours after the initial incubation.

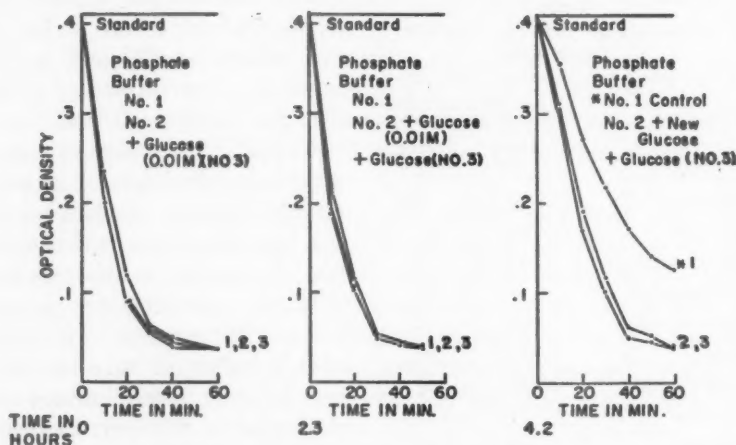
Secondly, this system of measuring total dehydrogenase activity was also used to simulate injury to a specific morphologic component of the cells, in this case the membrane including the glucose transporting system. Although other investigators have had success utilizing uranyl nitrate to block glucose transport in yeast cells,<sup>3</sup> this agent in concentrations up to .01 M was largely ineffectual in Ehrlich tumor cells (Text-figure 2).

Thirdly, total dehydrogenase activity of the cell, when glucose was given as a substrate, was used as an index to the amount of radiation desired in the general experiments. As is shown in Text-figure 3, only  $1 \times 10^6$  r. was capable of producing any measureable effect immediately. Even  $3 \times 10^6$  r.

showed no effect if the reduction of methylene blue was measured only at the end of 2-hour periods. There was little immediate difference in the effect produced by  $7 \times 10^6$  and  $9 \times 10^6$  r.

In some experiments there was no immediate effect on the dehydrogenase activity, either of  $1 \times 10^6$  r. or of 0.001 M salyrgan (Text-figure 5). After a period of 1.8 hours incubation at 37.5° C., aliquots of the irradiated cells showed some loss of activity, but this was not completely lost for a period of 6 hours.

It was previously noted that control cells contained sufficient endogenous substrate and dehydrogenase activity to reduce methylene blue completely for 2.3 hours. Irradiated cells did not do so well unless glucose was added at 2 hours (Text-fig. 4). Contrary to the experi-

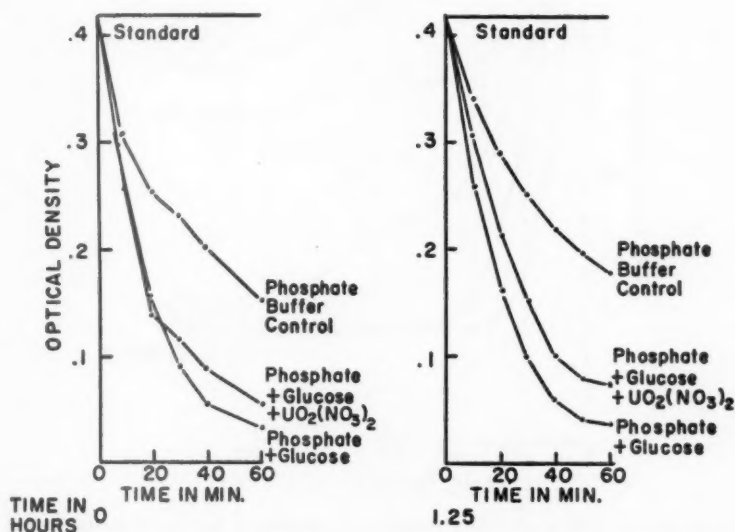


Text-figure 1. Effect of added substrate on the dehydrogenase activity of Ehrlich tumor cells. Amount of nonreduced methylene blue remaining is recorded as units of optical density. Each point represents the average results in 3 tubes. Three flasks containing cells were incubated at 37° C. for 4.2 hours and samples taken for Thunberg assay at the recorded times. Flask 3 was originally incubated with 0.01 M glucose and rapid reduction of the dye is noted after 4.2 hours. Flask 2 was incubated without glucose but this substrate was added to the Thunberg tube at 2.3 and 4.2 hours assay periods. A reduction in dye comparable to tube 3 is noted at all periods. Tube 1 never had glucose introduced and was completely dependent on the endogenous reserves of the cell. Only after 4.2 hours was a lag apparent in the reduction of the dye.

ments with control cells, which responded to exogenous glucose as late as 4.2 hours, the introduction of added glucose to the irradiated cells had no effect on the dehydrogenase activity after 4.1 hours.

The fourth and final use of the Thunberg tubes was to evaluate the effect of irradiation on dehydrogenase enzymes with SH groups. It has previously been reported that SH enzymes are more susceptible to irradiation than other enzymes.<sup>4</sup> By giving specific non-SH and SH substrates to starved Ehrlich tumor cells, it was possible to measure the activity of specific dehydrogenase enzymes following irradiation. Table II shows that the effect of irradiation on the SH enzymes, succinic dehydrogenase, pyruvic dehydrogenase, and glutamic dehydrogenase, was less than the effect of the non-SH enzyme, lactic dehydrogenase. It was also less than the effect on the oxidation

reduction enzymes acting when glucose was given as a substrate. However, the controls with substrates other than glucose were also markedly less affected.

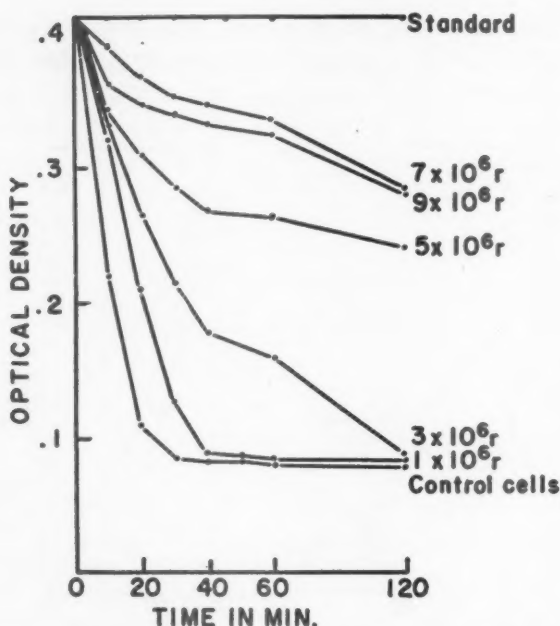


Text-figure 2. Effect of uranyl nitrate 0.01 M on the ability of starved Ehrlich tumor cells to utilize glucose as measured by dehydrogenase activity. Each point represents the average of 3 samples. Starved cells utilized exogenous glucose 0.01 M very noticeably when compared with cells given only phosphate buffer without glucose. Uranyl nitrate apparently had no effect on glucose utilization either immediately or after 1.25 hours incubation with the drug at 37° C.

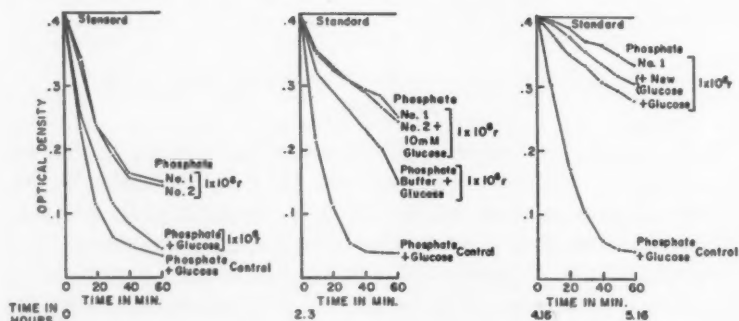
#### *The Effect of Injury on Respiration and Lactic Acid Production*

Studies on the respiration of Ehrlich tumor cells were carried out simultaneously with the other procedures. It was found initially that the respiration of a given number of cells ( $15 \times 10^6$ ) did not vary appreciably, regardless of the volume in which they were placed.

The endogenous substrates of Ehrlich tumor cells proved to be sufficient to maintain the cells for slightly over two hours. In accord with other investigators, it was found that the use of concentrations of glucose greater than 0.0055 M proved to be inhibitory to respiration<sup>5</sup> (Text-fig. 6). The only other substrate used besides glucose was a similar concentration of pyruvate (0.0014 M). This proved to be fully as capable as glucose in stimulating respiration.



Text-figure 3. Effects of massive doses of x-irradiation on the dehydrogenase activity of Ehrlich tumor cells. Each point represents the average of 3 samples in a typical experiment. Cells were immediately placed in Thunberg tubes after being given the various doses noted and the reduction of methylene blue recorded over a 2-hour period.



Text-figure 4. Effect of irradiation on the ability of Ehrlich tumor cells to utilize added glucose (0.01 M). Four Erlenmeyer flasks containing cells were incubated at 37° C. At the stated time periods, triplicate samples were removed for dehydrogenase assays in Thunberg tubes. Flasks 1, 2 and 3 contained cells previously irradiated, while the bottom line (flask 4) represents the control. Flask 1 was kept in a phosphate buffer and given no added glucose. Flask 2 was given added glucose after 2.3 and 4.16 hours of incubation in phosphate buffer. Flask 3 was given added glucose in the original incubation medium, as was the control Flask 4. Compare with Text-figure 1.

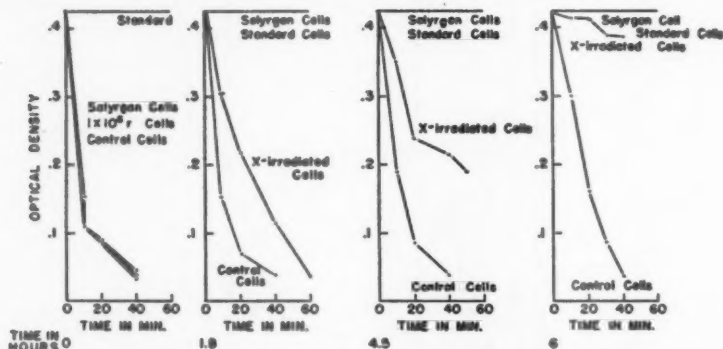
TABLE II  
Effect of Varying Doses of X-irradiation on Dehydrogenase Activity of Oxidation-Reduction Enzymes, Including 3 Enzymes Containing SH Groups

| Per cent of non-reduced methylene blue at end of one hour and per cent of inhibition of activity |         |       |                |        |       |               |         |       |               |          |       |               |          |       |               |
|--|---------|-------|----------------|--------|-------|---------------|---------|-------|---------------|----------|-------|---------------|----------|-------|---------------|
| SH enzymes   |         |       |                |        |       |               |         |       |               |          |       |               |          |       |               |
| Exper.<br>no.  | Glucose |       |                | Lactic |       |               | Pyruvic |       |               | Succinic |       |               | Glutamic |       |               |
|  | Cont.*  | X-ray | Inhib.†<br>(%) | Cont.  | X-ray | Inhib.<br>(%) | Cont.   | X-ray | Inhib.<br>(%) | Cont.    | X-ray | Inhib.<br>(%) | Cont.    | X-ray | Inhib.<br>(%) |
|  |         |       |                |        |       |               |         |       |               |          |       |               |          |       |               |
| 1  | 15      | 81    | 66             | 24     | 89    | 65            | 31      | 85    | 54            | 35       | 78    | 43            | 25       | 42    | 17            |
| 2  | 11      | 88    | 77             | 29     | 92    | 63            | 32      | 86    | 54            |          |       |               | 24       | 79    | 55            |
| 5  | 11      | 96    | 85             | 3      | 93    | 90            | 22      | 85    | 63            | 47       | 74    | 27            |          |       |               |
| 7  | 7       | 80    | 73             | 16.3   | 89    | 72.7          | 34      | 29    | 5             | 39       | 47    | 8             |          |       |               |

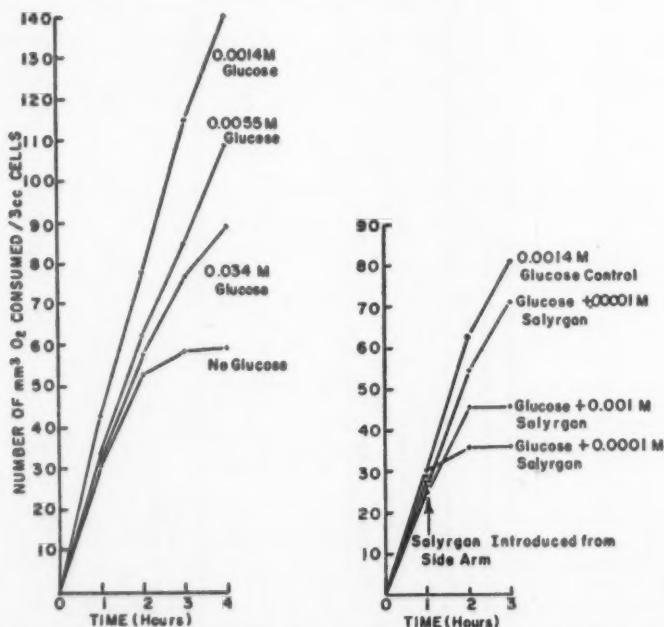
\* Cont. = Control

† Inhib. = Inhibition

Several specific chemical inhibitors of the glycolytic cycle were used in an attempt to block specific parts of the cycle and thus simulate injury by other means. The Ehrlich tumor cells appeared resistant to

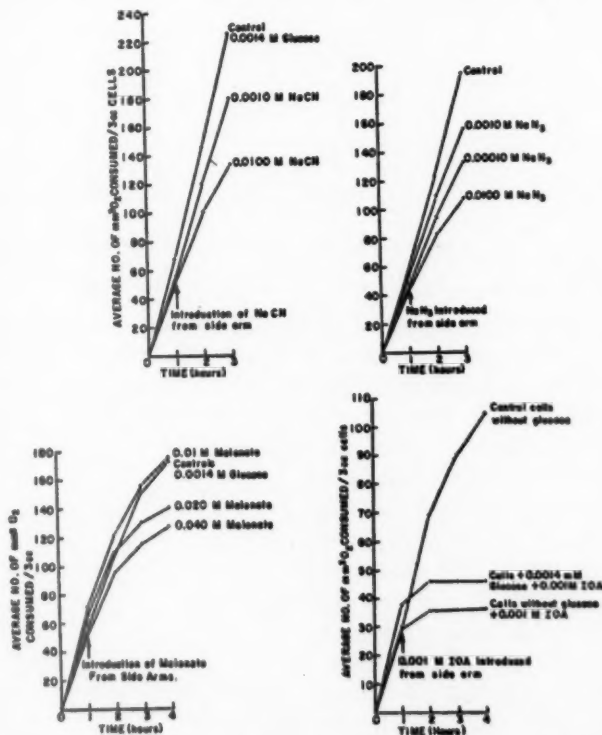


Text-figure 5. Effect of added substrate on the dehydrogenase activity of irradiated tumor cells. Control irradiated and salyrgan-treated cells were incubated in Erlenmeyer flasks with glucose 0.01 M at 37° C. At the stated time periods, samples were removed in triplicate and dehydrogenase assays run in Thunberg tubes. A difference among the 3 samples is noted at the first time period of 1.8 hours.



Text-figure 6. Inhibitory action of glucose and salyrgan on the respiration of Ehrlich tumor cells. Each point represents the average of 3 Warburg vessels.

several inhibitors previously found to be effective in other tissues. Salyrgan, a mercurial inhibitor of SH enzymes, was used in concentrations ranging from 0.001 to 0.010 M as a control inhibitor which would completely abolish energy formation in the cell (Text-fig. 6). Malonate in concentrations of 0.001 to 0.040 M had little effect during a 1-hour incubation period. Cyanide in concentrations of 0.001 to 0.010 M was similarly ineffectual during a 1-hour incubation period. Azide in concentrations of 0.001 to 0.010 M was ineffective during a

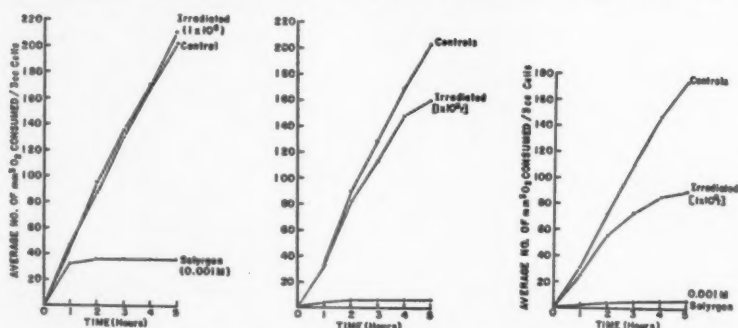


Text-figure 7. Effect of several metabolic inhibitors in various concentrations on the respiration of Ehrlich tumor cells. Each point represents the average of 3 Warburg flasks.

1-hour incubation period. Iodoacetamide was effective in concentrations of 0.001 M. Its effect was more pronounced in those cells incubated without glucose. In a later experiment, it was found to inhibit not only the fermentative part of the glycolytic cycle, but also the oxidative part (Text-fig. 7). The only specific inhibitor found for one particular part of the cycle was nitrogen.



The process of respiration in cells has long been noted for its resistance to radiation. In 60 cc. suspensions of Ehrlich tumor cells receiving average dosages of 501,000 r., there was no inhibition of respiration over a 5-hour period. As the volume of suspended cells was decreased, the average dose received by the cells increased. Thus, when



Text-figure 8. Effect of irradiation on the respiration of Ehrlich tumor cells. Effective dose of radiation received by the cells (after correction for varying depths of solution) is from left to right; 501,000 r., 668,000 r., and 794,000 r. respectively.

the suspension was reduced to 40 cc., the average dose received was 668,000 r. Although this resulted in some inhibition after 2 hours, the effect was not significant until after 5 hours. However, a 25 cc. suspension receiving a surface dose of  $1 \times 10^6$  and an average dose of 794,000 r. showed a 50 per cent depression in respiration, with the effect starting after the first hour (Text-fig. 8).

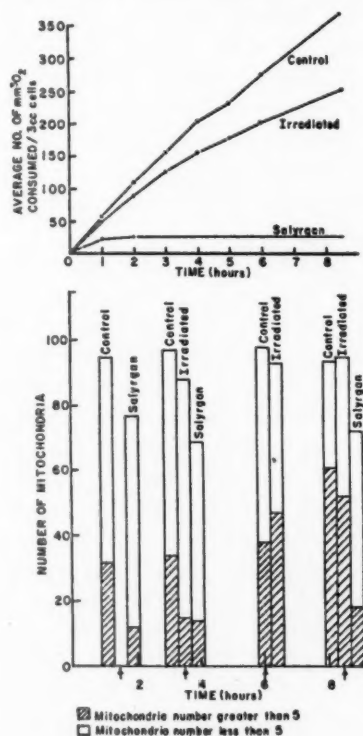
In experiments in this laboratory, lactic acid determinations on Ehrlich tumor cells were done on samples incubated both aerobically and anaerobically. As was to be expected, the cells were consistently able to produce more lactic acid when placed under nitrogen than when incubated with oxygen present. The degree varied from 11 to 28 per cent (Table III).

Caspersson<sup>6,7</sup> has reported on the inverse relationship between the size of the mitochondria and metabolic activity. Sarachek and Townsend<sup>8</sup> reported disruption of mitochondria by ultraviolet radiation. They found that swelling, disruption, and coalescence of these

TABLE III  
Comparative Amounts of Lactic Acid  
Produced by Ehrlich Tumor Cells  
Under Aerobic and Anaerobic Conditions

| Experiment no. | Micrograms of lactic acid |                      |                |
|----------------|---------------------------|----------------------|----------------|
|                | Aerobic incubation        | Anaerobic incubation | Inhibition (%) |
| 1              | 78                        | 88                   | 11.4           |
| 2              | 48                        | 55                   | 12.7           |
| 3              | 63                        | 82                   | 23             |
| 4              | 50                        | 60                   | 16.6           |
| 5              | 52                        | 73                   | 28.8           |

structures occurred before cytoplasmic proteins were precipitated. Salyrgan-treated cells showed large, swollen mitochondria centered around the shrunken nucleus. Schrek and Ott<sup>9</sup> noted the appearance of a granular crescent in the cytoplasm surrounding the nuclei of irradiated lymphocytes which might represent similar swollen mitochondria. It is very difficult to make accurate counts of mitochondria as they appear in different planes throughout the entire thickness of the cytoplasm. However, it was found that over 95 per cent of the control Ehrlich tumor cells had one or more visible mitochondria at the start of the experiment. Thirty per cent of these had 5 or more. Only 75 per cent of the salyrgan-treated cells had any mitochondria 2 hours after incubation, and less than 15 per cent had as many as 5 (Text-fig. 9).



Text-figure 9. Effect of salyrgan and irradiation on number of mitochondria in Ehrlich tumor cells. Cells were incubated for a period of 8 hours and respiration recorded in the top portion of the graph. At the stated times, cells were removed, stained for mitochondria, and the number of cells containing mitochondria (per 100 cells counted) recorded. Cells were further classified as to those containing more or less than 5 visible mitochondria at the stated time periods.

## DISCUSSION

It has been shown by several investigators that Ehrlich cells have a large endogenous carbohydrate reserve.<sup>5,10</sup> We have merely confirmed this by showing that the addition of glucose did not accelerate the reduction of methylene blue by Ehrlich cells until after two hours of incubation.

The Thunberg method is a convenient and easy way to measure total dehydrogenase activity. However, measurement of individual enzyme activities even in starved cells exhausted of endogenous substrates is subject to error because of the large number of substrates present and the rapidity of reaction rates.

The inhibitory effect of excessive amounts of glucose has been demonstrated in other laboratories, and glucose and pyruvate have been shown to possess the same relative effectiveness as substrates.<sup>5</sup> Kun, Talalay and Williams-Ashman<sup>10</sup> reported a linear respiration curve for 5 hours and 75 per cent depression in 11 hours. In most of our experiments, respiration remained linear for the duration of the experiments, and in one case this continued for a period of 18 hours. We believe this is due to the comparatively low cell concentration in our samples ( $5 \times 10^6$  per ml.).

Since Warburg,<sup>11</sup> Shade,<sup>12</sup> and others had stressed the anaerobic metabolism of Ehrlich tumor cells, it seemed important to investigate the ability of cells to perform in a normal manner when the anaerobic and aerobic portions of the glycolytic cycle were individually inhibited. Salyrgan (a mercurial inhibitor) proved very effective in inhibiting both lactic acid production and respiration in a concentration of 0.001 M. It proved to be impractical to inhibit the oxidative cycle with chemical inhibitors. Kun and co-workers<sup>10</sup> found that sodium cyanide 0.01 M and sodium azide 0.01 M (inhibitors of cytochrome oxidase) inhibited the endogenous respiration of Ehrlich cells 96 and 75 per cent respectively. Black, Kleiner, and Speer<sup>13</sup> had previously reported that sodium malonate 0.001 M had little or no effect on the dehydrogenase activity of mouse and human carcinomas. Our experiments showed that malonate 0.01 M, cyanide 0.01 M and azide 0.01 M had little if any immediate effect on the respiration of Ehrlich tumor cells. Higher concentrations were not used because the utilization of chemical agents as specific inhibitors of the energy producing reactions alone has been subject to criticism. For example, Parpart and Green<sup>14</sup> stated that some inhibitors (i.e., *p*-chloro-mercuro-benzoic acid) had a lytic effect on the membrane; thus they might affect electrolyte transfer for this reason and, secondarily, by decreasing energy production.

It also proved impossible to inhibit the anaerobic phase of glycolysis without interfering with the oxidative aerobic cycle. Iodoacetate 0.001 M, an inhibitor of glyceraldehyde dehydrogenase, and sodium fluoride 0.001 M, an inhibitor of enolase, both inhibited respiration as well as fermentation. It was, therefore, necessary to inhibit the Krebs cycle with a nitrogen atmosphere.

Kun and associates<sup>10</sup> reported that Ehrlich cells produced 19.7 M lactic acid under anaerobic conditions as compared with 14.3 M under aerobic conditions. This is a depression of approximately 17 per cent. Lactic acid production in tumor cells in this laboratory was depressed 11 to 28 per cent under aerobic conditions, as compared with the anaerobic experiments.

Boell<sup>15</sup> has reviewed some of the work on the effect of radiation on respiratory metabolism. Boell, and later Tahmisian<sup>16</sup> irradiated grasshopper eggs with dosages ranging from 25,000 to 200,000 r. Only with the strongest doses was a depression of respiration noted. Experiments in this laboratory were performed uniformly with  $1 \times 10^6$  r. surface dose. However, the amount of radiation one average cell received varied from 501,000 r. in a 60 cc. cell suspension to 794,000 r. in a 25 cc. suspension. This usually made a decided difference in the respiratory response of the cells. While the dose received by the cells in a 60 cc. suspension (501,000 r.) did not produce any depression of respiration, in 5 hours, a cell suspension of 40 cc. receiving an average dose of 668,000 r. showed a depression of 20 per cent in respiration, starting 3 hours after incubation. An average dose of 794,000 r. lowered respiration after one hour with a final  $Q_{O_2}$  approximately 50 per cent of the control cells.

Although Barron, Dickman, Muntz and Singer<sup>17</sup> reported inhibition of several enzymes by irradiation *in vitro*, other workers have failed to substantiate this.<sup>18-21</sup> The delayed-effect phenomenon may explain part of the reasons for the controversial findings. When a semipurified enzyme is isolated immediately following irradiation, it may show very little damage when tested in a triphosphopyridine nucleotide system spectrophotometrically. However, the time required for manometric techniques will permit secondary inhibitory effects to take place. Examples of this delayed-effect phenomenon have been found by Anderson<sup>22</sup> working with pepsin and by other workers utilizing trypsin.<sup>23-25</sup>

In experiments in this laboratory, the delayed effect of irradiation on dehydrogenase systems of Ehrlich tumor cells was very apparent. When specific substrates such as succinic, glutamic and pyruvic acid

requiring SH dehydrogenases were given to starved tumor cells irradiated with multiple dosages, there was usually less inhibition of activity than when substrates such as lactic acid requiring non-SH dehydrogenase were administered. While it seems likely from the observations of others that certain enzymes are more sensitive than others, there has not yet been sufficient work to enumerate the sensitive ones. Other groups, in addition to those in lactic dehydrogenase, may be equally or more sensitive to the deleterious effects of irradiation.

#### SUMMARY

Ehrlich tumor cells, when injured, showed no immediate diminution in glucolytic cycle activity and massive doses of irradiation were necessary to produce delayed damage in the intact cell. Cell respiration, lactic acid production, and total dehydrogenase activity were reduced many hours after the initial injury.

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## ACID MUCOPOLYSACCHARIDE GRANULES IN THE GLOMERULAR EPITHELIUM IN GARGOYLISM\*

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The disorder variously referred to as Hurler's or Hurler-Pfaundler's disease, dysostosis multiplex, lipochondrodystrophy, and gargoylism is clinically characterized by disproportionate dwarfism, skeletal deformity, grotesque facial appearance, hepatosplenomegaly, umbilical and inguinal herniation, corneal cloudiness, and mental retardation.<sup>1-6</sup> In this condition, dystrophic substance is stored in neuronal bodies of the central and autonomic nervous systems, in hepatic cells, endothelial cells of the splenic sinuses and pulmonary alveoli, in the connective tissue of cardiac valves and the endocardium, in cartilage cells of the spine and other bones, and in Bowman's layer of the cornea.<sup>4-11</sup> A variety of investigations concerning the chemical nature of the stored materials in gargoylism have been reported. Although a glycolipid was reported by some investigators,<sup>12-14</sup> knowledge accumulated during the last 6 years (since Brante,<sup>15</sup> 1952) indicates that the dystrophic substances in neurons of the central and autonomic nervous systems is histochemically similar to that encountered in Tay-Sachs<sup>5,10,16,17</sup> and Niemann-Pick diseases,<sup>18</sup> and is chemically identifiable as a ganglioside.<sup>10,15</sup> Recent studies also indicate that the dystrophic substance in visceral organs and connective tissues is histochemically and chemically identifiable as an acid mucopolysaccharide.<sup>14,15,18-21</sup>

On the basis of the "one-gene, one-enzyme" theory of modern genetics, gargoylism can be considered as a genetically determined generalized metabolic disease or enzymopathy. Since blood cells are rich in enzymes, the so-called "Reilly bodies"<sup>4</sup> or Alder's granulation anomaly in the leukocytes of patients with gargoylism have been referred to as one manifestation of generalized enzymopathy.<sup>11,22,23</sup> Hyperaminoaciduria has been observed in patients with this condition who showed no "Reilly bodies" in leukocytes.<sup>24</sup> However, aminoaciduria has been observed in many metabolic diseases, and is often believed to be a result of a genetically determined abnormality of

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tubular function.<sup>25</sup> More recently, hypermucopolysacchariduria has been reported in gargoylism.<sup>20,26</sup> This observation appears to be important, not only in the study of gargoylism itself, but also in the investigation of urinary manifestations of metabolic diseases in general.

Accordingly, an attempt was made to investigate the existence of histologic and histochemical alterations demonstrable in the kidneys of patients with gargoylism. In July, 1956, the writer observed small basophilic and PAS-positive granules in the glomerular epithelium of one patient (case 1). Lindsay, Reilly, Gotham and Skahen<sup>6</sup> had described pale gray granular deposits in the intercellular portions of epithelial crescents of some glomeruli in one of their patients (case 3). Since the kidney in our first case contained no crescents at all and the granules were located within the epithelial cells of the glomeruli, they were considered to be different from those described by this group of investigators. Seitelberger also described glycolipid storage nephrosis, similar to that of metachromatic leukodystrophy, in patients with gargoylism,<sup>12,13</sup> but none of our patients showed this type of lesion. The present communication is concerned with the histochemical nature of the granules in the epithelial cells of the glomeruli in gargoylism. Particular emphasis is given to their significance in the pathogenesis of gargoylism, and to the possibility of clinical application.

#### MATERIAL AND METHODS

Tissue was available from two patients (cases 1 and 2) at the Cincinnati Children's Hospital, one patient (case 3) at the Cincinnati General Hospital, and 5 patients (cases 4, 5, 6, 7 and 8) at the Boston Children's Medical Center. All the patients were recognized clinically as having gargoylism, and the diagnosis was confirmed by pathologic examination. One patient with acute laryngotracheitis, but otherwise with no significant ailment, was utilized as a control.

The kidney tissues used for this study were fixed in either Zenker's acetic acid solution, formalin, or Lindsay's dioxane fixative.<sup>6</sup> They were dehydrated through ethanol to chloroform, and embedded in Fisher Tissuemat. Sections were cut at two  $\mu$  thickness. As survey stains, hematoxylin and eosin and periodic acid-Schiff stains were applied.

The sex and age of the patients so far as is known, names of the institutions from which the tissues were obtained, and the results of the survey stains are listed in Table I.

#### RESULTS

The glomerular granules were well demonstrated by the survey stains in Zenker-fixed tissue, but not in that which was formalin-fixed.

They were poorly visualized in Lindsay-fixed tissue. The PAS stain was carried out with both aqueous and alcoholic solutions of periodic acid; the alcoholic solution was found to be more effective in that the granules were more intensely stained.

TABLE I  
*Demonstration of Glomerular Granules*

| Case no. | Age         | Sex     | Institution | Year | Fixing solution and stain |     |         |     |          |     |
|----------|-------------|---------|-------------|------|---------------------------|-----|---------|-----|----------|-----|
|          |             |         |             |      | Zenker                    |     | Lindsay |     | Formalin |     |
|          |             |         |             |      | H&E                       | PAS | H&E     | PAS | H&E      | PAS |
| 1        | 5 1/12 yrs. | F       | CCH         | 1956 | +                         | +   | ±       | +   | —        | —   |
| 2        | 2 1/2 mos.  | F       | CCH         | 1956 | —                         | +   | —       | —   | —        | —   |
| 3        | 4 yrs.      | F       | CGH         | 1957 | +                         | +   | —       | —   | —        | —   |
| 4        | 6 yrs.      | Unknown | BCMC        | 1954 | —                         | —   | —       | —   | —        | —   |
| 5        | 12 yrs.     | Unknown | BCMC        | 1954 | —                         | +   | —       | —   | —        | —   |
| 6        | 3 3/12 yrs. | Unknown | BCMC        | 1957 | —                         | —   | —       | —   | —        | —   |
| 7        | 9 1/12 yrs. | Unknown | BCMC        | 1949 | —                         | —   | —       | —   | —        | —   |
| 8        | 5 9/12 yrs. | Unknown | BCMC        | 1949 | —                         | —   | —       | —   | —        | —   |
| Control  | 5 5/12 yrs. | F       | CCH         | 1958 | —                         | —   | —       | —   | —        | —   |

CCH = Cincinnati Children's Hospital

CGH = Cincinnati General Hospital

BCMC = Boston Children's Medical Center

± = well defined granules present

± = granules questionable and scanty

The Zenker-fixed tissues from Boston Children's Medical Center had been stored in alcohol, and the formalin-fixed tissues in formalin. The tissues of the Cincinnati patients were fixed at the time of necropsy and preserved in paraffin.

Granules were located in the epithelial cells of glomerular tufts, occasionally attached to the nuclei (Figs. 1 to 4). They were irregular, usually appeared clumped, and measured up to one-half a red cell diameter (3 to 4  $\mu$ ). From a practical standpoint, the granules were not only beautifully demonstrated by the PAS stain, but also by the May-Grünwald Giemsa stain and by toluidine blue at pH 4 to 5.

In one patient (case 3), the granules were noted to be limited to the juxtamedullary glomeruli; in other cases the distribution was rather uniform in all glomeruli. This distribution is interesting in comparison with that of Armani-Ebstein diabetic nephropathy. Granules in the latter condition usually appear in nephrons of the outer medulla and inner cortex.<sup>29</sup> It is known that at birth the glomeruli of the inner cortex are histologically more mature or differentiated than those of the outer cortex. Therefore, one can speculate that development of the glomerular granulation depends on the duration of the disease; i.e., the age of the patient. However, the dis-

tribution of the granules in relation to age has not been evaluated because of the limited number of cases.

The tissues from the Boston Children's Medical Center contained practically no granules. This is considered to be due to the method of storage of the tissues, a factor to be discussed later.

In order to investigate the failure to demonstrate granules in the formalin-fixed tissue, the effects of the various steps utilized in advance of staining of Zenker-fixed tissue were considered. The formalin-fixed tissues of one patient (case 1) were treated by zenkerization, iodination, and reduction by sodium thiosulfate, in the following combinations, and then stained by the alcoholic PAS stain. Lindsay-fixed tissue was subjected to the same procedures.

Formalin-fixed tissue + zenkerization + alcoholic PAS.

Formalin-fixed tissue + zenkerization + iodination + sodium thiosulfate reduction + alcoholic PAS.

Formalin-fixed tissue + zenkerization + iodination + alcoholic PAS.

Lindsay-fixed tissue + zenkerization + alcoholic PAS.

Lindsay-fixed tissue + zenkerization + iodination + sodium thiosulfate reduction + alcoholic PAS.

Lindsay-fixed tissue + zenkerization + iodination + alcoholic PAS.

No significant changes were demonstrated, except that both iodination and sodium thiosulfate reduction significantly accentuated the PAS positive reaction in the granules preserved in Lindsay-fixed tissue. This indicated that formalin fixation was not a suitable method for preservation of the granules. Zenker fixation yielded the best results, and Lindsay's fixation was next. Since the granules tolerated ordinary staining procedures, it was apparent that alcohol and other organic solvents did not dissolve them. The water in formalin fixative was, therefore, considered to be responsible for the failure of granule preservation in tissue fixed by this means. The greater effectiveness of alcoholic PAS stain, as compared to that of the aqueous stain, supported this opinion. The failure to demonstrate granules in the Zenker-fixed tissues from the Boston Children's Medical Center was also considered attributable to dissolution of the granules. On the other hand, the possibility that they were not present in some of the patients is undeniable.

For investigation of the chemical nature of the granules, various histochemical procedures were applied; these can be categorized in 6 groups as procedures for demonstration of carbohydrates, lipids and proteins, enzymatic digestion, pH extinction, and miscellaneous pro-

cedures. These procedures and their results are tabulated in Table II. Examined under polarized light and by dark field, the granules exhibited no refractility.

On the basis of the histochemical investigations, the glomerular granules were considered to contain acid mucopolysaccharide, similar to chondroitin sulfate and heparin. Since similar granules were not demonstrated in the glomeruli of the control case, they apparently represented a "dystrophic" substance. There was no evidence that they contained protein, nucleoprotein, or lipid. The possibility that they constituted nuclear debris was ruled out by the negative Feulgen reaction. As indicated by toluidine blue extinction and staining with the Hale, acridine orange, Alcian blue, aldehyde fuchsin, and the May-Grünwald Giemsa stains, it would appear that the substance comprised large numbers of acid radicals or valences. Since acetylation did not block the PAS positive reaction significantly, and on the assumption that alkyl amino groups are less easily acetylated than 1,2-glycols or 1-amino 2-hydroxy groups, it is possible that large numbers of alkylamino groups were present.<sup>30</sup> Deacetylation by potassium hydroxide decreased the PAS positivity significantly, presumably because the granules were dissolved by the alkaline medium. The effect of thioglycolate can also be understood on this basis, since it was used in alkaline solution. The enzyme digestions applied did not affect the granules except in the case of testicular hyaluronidase. Although digestion by hyaluronidase was not complete, it was sufficient to suggest that chondroitin sulfate A or C, or hyaluronic acid was present in the granules.

In an effort to produce the granules experimentally, a fragment of normal kidney was soaked in heparin sodium solution (1,000 units per cc.) for 30 minutes; similar granules were not demonstrated in toluidine blue stained sections. Sections of Zenker-fixed tissue from the brain, lung, liver, spleen, pituitary, thyroid, adrenal and gastrointestinal tract from one patient (case 1) were stained by the alcoholic PAS method, but no granules were apparent. Unfortunately, tissue from the choroid plexus was not available for examination; lipid-containing granules have been observed in this location in gargoylism.<sup>19</sup>

#### DISCUSSION

The granules encountered in renal glomerular epithelium in patients with gargoylism are considered to constitute additional support for the theory implicating a generalized enzymopathy in this disease. This is especially so since patients with this disease are known to

exhibit mucopolysacchariduria. Dorfman and Lorincz,<sup>28</sup> Grumbach and Meyer,<sup>29</sup> and Linker, Hoffman and Meyer<sup>31</sup> have demonstrated two types of mucopolysaccharides in the urine in gargoylism, a relatively large amount of chondroitin sulfate B (beta heparin) and a small amount of heparin monosulfuric acid<sup>28</sup> or heparitin sulfuric acid.<sup>29</sup> Since Brante's report in 1952,<sup>15</sup> the dystrophic substance found in visceral organs has been chemically and histochemically identified as an acid mucopolysaccharide.<sup>14,18-20</sup> That in the neurons of the central nervous system has been found to be similar to the substance in Tay-Sachs or Niemann-Pick diseases, and has been chemically identified as ganglioside.<sup>6,10,12,14,18</sup> The latter observation is the subject of some controversy, however, since Bishton, Norman and Tingey reported no significant elevation of cerebroside or ganglioside in the central nervous system.<sup>19</sup>

An effort at identification of the chemical nature of the dystrophic substance in the visceral organs has been made by various investigators. Uzman isolated two substances from the spleen and liver of

TABLE II  
*Histochemical Characteristics of Glomerular Granules*

| Methods for demonstration                            | Response of granules |
|--|----------------------|
| 1. Carbohydrates in general, and mucopolysaccharides |                      |
| PAS  | ++                   |
| Alcoholic PAS  | ++++                 |
| Iodination + alcoholic PAS                           | +++++                |
| Na thiosulfate reduction + alcoholic PAS             | +++ to ++++          |
| Acetylation + alcoholic PAS                          | ++++                 |
| Acetylation, KOH + alcoholic PAS                     | +                    |
| Diezel neuraminic acid stain (Bial's)                | +                    |
| Toluidine blue for metachromasia                     | +++++                |
| Thioglycolate + toluidine blue                       | —                    |
| Hale stain   | ++                   |
| Alcian blue  | +++                  |
| 2. Proteins and nucleoproteins                       |                      |
| Millon reaction                                      | —                    |
| Coupled tetrazonium                                  | —                    |
| Dihydroxydinaphthyl disulphide                       | —                    |
| Thioglycolate + dihydroxydinaphthyl disulphide       | —                    |
| Direct HCl-Schiff (Feulgen)                          | —                    |
| Iodination-Schiff reaction                           | —                    |
| Bromination-Schiff                                   | —                    |
| Na thiosulfate reduction-Schiff                      | —                    |
| 3. Lipids  |                      |
| Sudan IV   | —                    |
| Sudan black  | —                    |
| Luxol blue   | —                    |
| Direct Schiff stain                                  | —                    |
| Phosphomolybdic acid-stannous chloride               | —                    |
| Performic Schiff stain                               | —                    |
| Pyridine extraction                                  | —                    |



## 4. Toluidine blue extinction

| pH  | Basement membrane |      |
|-----|-------------------|------|
| 2   | —                 | +++  |
| 3   | —                 | +++  |
| 4   | —                 | +++  |
| 4.5 | +                 | ++++ |
| 5   | ++                | ++++ |
| 6   | ++                | ++++ |
| 7   | ++                | ++++ |

## 5. Enzyme digestion

|  |   |
|--|---|
| Saliva, human, 30 minutes  | No digestion  |
| Pectinase, 100 mg. per 100 cc., pH 4, $\times$ 1 hour  | No digestion  |
| Glucuronidase (Ketodase, Warner-Chilcott Co.)<br>250,000 units per 50 cc., pH 5, $\times$ 1 hour   | No digestion  |
| Malt diastase, 100 mg. per 100 cc., pH 6,<br>$\times$ 1 hour                                       | No digestion  |
| Testicular hyaluronidase, (Wydase), 150 turbidity<br>reducing units per cc., pH 6, $\times$ 1 hour | Slight digestion;<br>significant decrease<br>in number and size |

## 6. Miscellaneous

|   |                              |
|---|------------------------------|
| Phosphotungstic acid hematoxylin stain                              | —                            |
| Direct PTAH   | —                            |
| Aldehyde fuchsin stain  | ++                           |
| Acid $\text{KMnO}_4$ + aldehyde fuchsin stain                       | +++                          |
| Iodination + aldehyde fuchsin stain                                 | +++                          |
| Na thiosulfate reduction + aldehyde fuchsin<br>stain                | +                            |
| Iodination + Na thiosulfate reduction + alde-<br>hyde fuchsin stain | ++                           |
| Thioglycolate + aldehyde fuchsin stain                              | $\pm$                        |
| Thioglycolate + acid $\text{KMnO}_4$ + aldehyde<br>fuchsin stain    | +                            |
| May-Grünwald-Giemsa stain   | +++<br>(azurophilic)         |
| Acridine orange   | +++<br>(orange fluorescence) |

gargoylism, a complex polysaccharide (Fraction P) and a glycolipid (Fraction S).<sup>14</sup> Brown characterized the storage substance as an oligosaccharide.<sup>21</sup> Grumbach and Meyer<sup>20</sup> and Linker and co-workers<sup>31</sup> isolated heparitin monosulfate from the liver and spleen. These substances are compared with each other in Table III.

The acid mucopolysaccharide,<sup>15</sup> Fraction P,<sup>14</sup> and chondroitin sulfuric acid B<sup>20,26</sup> can be considered to be chemically identical or similar. The oligosaccharide,<sup>21</sup> heparin monosulfuric acid<sup>26</sup> and heparitin sulfate<sup>20</sup> may also represent identical or similar substances. However, the Fraction S demonstrated by Uzman<sup>14</sup> appears to be unique; he considered it to be a glycolipid. This substance may have some relationship to the dystrophic substance found in the central nervous system. Seitelberger theorized that gargoylism was a glycolipidosis rather than a mucopolysaccharidosis.<sup>12,13</sup>



TABLE III  
*Gargoylism*  
*Chemically Identified Dystrophic Substance in the Visceral Organs and Urine*

| Investigators  | Location           | Chemical entity                                       | Solubility   | Chemical constituents  |
|--|--------------------|---|--|--|
| Brante (1952) <sup>16</sup>  | Liver and spleen   | An acid mucopolysaccharide                            | Soluble in water.  | (?) Chondroitin and sulfuric acid (hexosamine present).  |
| Uzman (1955) <sup>14</sup>   | Liver and spleen   | A complex polysaccharide (Fraction F)<br>A glycolipid | Soluble in water and formalin. Insoluble in ethanol, methanol and other organic solvents.<br>Soluble in water and ethanol. | Glucose, galactose, hexosamine, sulfate.<br>Fatty acid, sphingosine, neuraminic acid, hexuronic acid, hexosamine, glucose, galactose.  |
| Brown (1957) <sup>21</sup>   | Liver and spleen   | An oligosaccharide                                    | Soluble in water.  | D-glucosamine (acetylated and sulfated), D-glucuronic acid.  |
| Grumbach and Meyer (1958) <sup>20</sup> ; and Linker, Hoffman and Meyer (1958) <sup>21</sup> | Liver<br><br>Urine | Heparitin sulfate<br><br>Heparitin sulfate            | Soluble in water.<br><br>Soluble in water.   | N-acetylated glucosamine, hexuronic acid. Sulfate Mol/Mol repeating unit $\leq 1$ .<br><br>N-acetylated glucosamine, hexuronic acid. Sulfate Mol/Mol repeating unit $\leq 1$ . |
|  | Urine              | Chondroitin sulfate B                                 | Soluble in water.  | Galactosamine, iduronic acid, sulfate Mol/Mol repeating unit = 1.  |
| Dorfman and Lorincz (1957) <sup>20</sup>   | Urine              | Chondroitin sulfate B                                 | Soluble in water.  | Galactosamine, iduronic acid, sulfate Mol/Mol repeating unit = 1.  |
|  | Urine              | Heparin monosulfate                                   | Soluble in water.  | D-glucuronic acid, D-glucosamine sulfate Mol/Mol repeating unit = 1.   |

On a histochemical basis, the granules observed in the present cases cannot be considered identical to any single one of the substances cited (i.e., chondroitin sulfuric acid B, heparin monosulfuric acid, or heparitin sulfate) because each of these exhibited the histochemical properties demonstrated in our studies. It is apparent, however, that the granule would not be related to the Fraction S of Uzman.

The pathogenesis of the granules is a matter of speculation. One might assume the existence of a hypermucopolysaccharidemia in gargoylism. Thus, during urine formation this substance might be deposited in the epithelial cells of glomeruli. Although the existence of hypermucopolysaccharidemia would not be strictly necessary for this to occur, such a state has not been demonstrated in gargoylism. The storage nephropathy discussed by Seitelberger,<sup>12,13</sup> in gargoylism characterized by deposition of metachromatic glycolipid in the epithelial cells of the distal portions of the renal tubules, was not demonstrated in our cases. If this were as definite and constant an occurrence in gargoylism as in the case of metachromatic leukodystrophy,<sup>27,28</sup> the pathogenesis of the tubular alteration might be considered to have a similar mechanism.

Paper chromatographic study has shown hyperaminoaciduria in patients with gargoylism whose blood contained no "Reilly bodies." The aminoaciduria was characterized by excretion of cysteic acid, glycine, serine, ethanol aminophosphoric acid (?), alanine, proline, valine, phenylalanine, tyrosine, tryptophan, glutamic acid, and aspartic acid.<sup>24</sup> However, the significance of this phenomenon is still debatable, since Berger,<sup>32</sup> and Bickel and Souchon<sup>33</sup> have failed to demonstrate hyperaminoaciduria in gargoylism, even in patients without "Reilly bodies." The substantiation of the observation of hypermucopolysacchariduria and hyperaminoaciduria in gargoylism would support the hypothesis that the glomerular granules were a consequence of chemical abnormality of the urine. This is especially so when one recognizes that the granules are not large enough to be a source for these substances in the urine.

As an alternative possibility, one could assume that the granules arose *in situ* as a manifestation of cellular dysfunction associated with the underlying enzymopathy. Since the enzymopathy in gargoylism would affect all tissues; i.e., the connective tissues, cartilage, bone, leukocytes, *tunica propria* of the cornea, sinusoidal cells of the lung, liver and spleen, it is conceivable that the granules might constitute the morphologic representation of the effect of the enzymopathy in the epithelial cells of the glomeruli as well. "Reilly bodies" or Alder's granules in the leukocytes might be considered to represent a similar

phenomenon. According to some investigators,<sup>22,23</sup> Alder's anomaly of granulation is a hematologic manifestation associated with "constitutional dysostosis enchondralis," occurring both in gargoylism and Morquio's disease. Similar bodies in the leukocytes of a patient with gargoylism were observed by Reilly in neutrophils, eosinophils, and monocytes; eosinophils were most strikingly affected.<sup>34</sup> Ullrich and Wiedemann considered that "Reilly bodies" were composed of a polysaccharide complex containing hyaluronic acid, presumably an acid mucopolysaccharide.<sup>22</sup> Kosenow and Wiedemann, on the other hand, concluded that they contained alcohol-extractable and Sudan black-positive lipid, with no significant difference from normal in the content of carbohydrate and nucleoprotein.<sup>23</sup> Since alcohol extractability and Sudan black positivity are not specific indications of lipid, Kosenow and Wiedemann's considerations justify some reservation. It is probable that the bodies might have some relation to the Fraction S described by Uzman or to the substance observed in ballooned Kupffer's cells. This has been described as neutral lipid by some investigators.<sup>11-13,35</sup> Diezel considered the "Reilly bodies" to be composed of polysaccharide, presumably mucopolysaccharide, with low grade of polymerization and slight water solubility.<sup>11</sup> The apparent difference in solubility of the glomerular granules and "Reilly bodies" may thus stem from different grades of polymerization. Although no proof is available, it is felt that the glomerular granules can reasonably be considered to represent a dystrophic substance formed *in situ* as the result of the basic enzymopathy. It is conceivable that the granules actually represent mitochondria containing the dystrophic mucopolysaccharide. This was proposed by Diezel in the case of "Reilly bodies."<sup>11</sup>

Generally speaking, gargoylism is accompanied by a variety of morphologic manifestations of the underlying metabolic disturbance, with each organ exhibiting lesions which may be chemically and physiologically different. For instance, the glomerular granules and the dystrophic substance in the connective tissues and reticuloendothelial cells both appear to be acid mucopolysaccharides, but the glomerular granules are best preserved by Zenker's solution, while the dystrophic substance in other tissues is preserved best by Lindsay's solution.

The histochemically demonstrable dualism in the dystrophic substance suggests that gargoylism should be placed in a category between the lipidoses, such as the Tay-Sachs and Niemann-Pick diseases, and the glycogen storage diseases, particularly the type characterized by

debranching enzyme deficiency. It is possible that an enzymopathy of a debranching or depolymerizing enzyme in gargoylism may result in an accumulation of such varied macromolecular compounds as acid mucopolysaccharide and ganglioside in different organs.

Two clinical forms of gargoylism have been reported. One appears to be the result of a simple autosomal recessive gene and is typical in its manifestations. The other is a sex-linked recessive type with milder clinical manifestations, lacking severe mental retardation and corneal cloudiness. The latter form has its onset at a later age, and is characterized by longer survival.<sup>7,86</sup> Since glomerular granules were not observed in all cases studied, a conclusion cannot be drawn concerning any relationship with these clinical types. However, it should be noted that the patients in whom the granules were observed (cases 1, 2 and 3) were all female, so that the disease here did not represent the recessive sex-linked type.

Establishment of the diagnosis of gargoylism is not difficult when the disease is fully manifested. The mild sex-linked form, however, can be confused with other skeletal disorders, notably Morquio's disease. In order to establish the diagnosis, biopsy of liver or spleen may be helpful,<sup>87</sup> particularly when the tissue is subjected to adequate histochemical analysis. Metachromatic substances have been demonstrated in both splenic and hepatic tissues in gargoylism.<sup>88</sup> Appendectomy or biopsy of rectal wall may be used for histochemical demonstration of the dystrophic deposit in ganglion cells of the myenteric plexus. It is also possible that renal biopsy may contribute to the establishment of the diagnosis when glomerular granules are demonstrable. It is possible that these granules may be more regularly present than "Reilly bodies" in the white cells. Since the urinary excretion of metachromatic granules or casts has been reported in metachromatic leukodystrophy,<sup>27,28</sup> this process should be considered in the differential diagnosis, even though the two diseases are clinically completely different. Histochemically, the deposit in gargoylism is red, while that in metachromatic leukodystrophy is golden brown when stained with toluidine blue. However, the dystrophic substances responsible for the renal tubular lesions are said to be glycolipids in both disorders.<sup>12,18,27,28</sup>

#### SUMMARY

Acid mucopolysaccharide granules were demonstrated in the epithelial cells of the glomeruli in 3 patients with gargoylism. Their occurrence was considered to represent an additional manifestation of the underlying enzymopathy affecting carbohydrate metabolism in gar-

goylism. The possible relation of the granules to hypermucopolysacchariduria and to "Reilly bodies" has been discussed, and certain clinical implications have been proposed.

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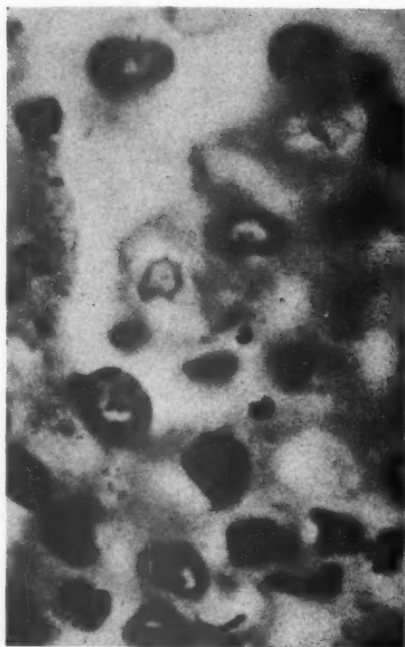
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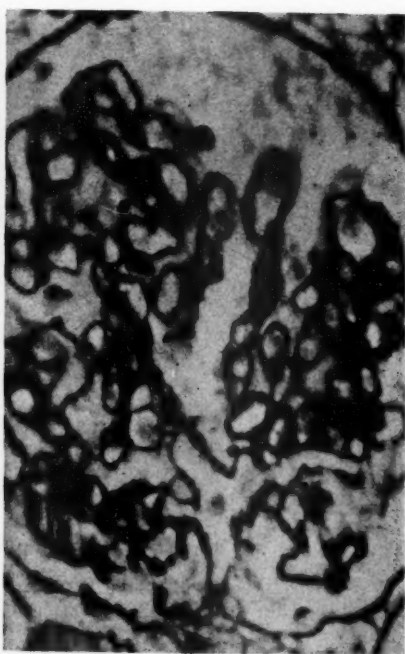
#### LEGENDS FOR FIGURES

- FIG. 1. Basophilic granules are visible in the epithelial cells of the glomerular tufts. Hematoxylin and eosin stain.  $\times 1,000$ .
- FIG. 2. Glomerular granules are PAS positive. Some of them appear aggregated. Periodic acid-Schiff stain.  $\times 450$ .
- FIG. 3. Glomerular granules prove to be aldehyde fuchsin positive. Acid  $\text{KMnO}_4$  aldehyde fuchsin stain.  $\times 450$ .
- FIG. 4. The granules are azurophilic; some appear to be attached to nuclei. May-Grünwald-Giemsa stain.  $\times 450$ .

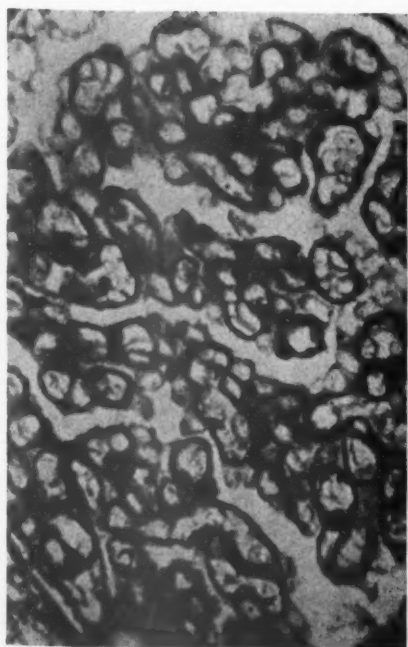




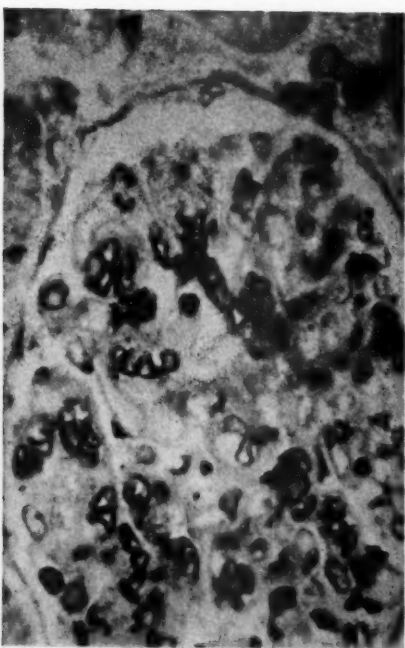
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HISTOCHEMICAL STUDIES OF ACIDOPHILIC, CRYSTALLINE  
INTRANUCLEAR INCLUSIONS IN THE LIVER  
AND KIDNEY OF DOGS\*

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Acidophilic, crystalline intranuclear inclusions have long been recognized in epithelial cells of the liver and renal cortex of dogs. They were first reported in 1902 by Browicz,<sup>1</sup> who observed what he thought were hemoglobin crystals in the nuclei of liver cells, while investigating the bile canaliculi of canine livers. A drawing by Browicz of the liver cell in dogs appeared the same year in MacCallum's translation of Szymonowicz's textbook of histology.<sup>2</sup> A crystal of rectangular parallelepiped shape, described as hemoglobin, was depicted within the nucleus.

In 1909, Brandts<sup>3</sup> reported "square," crystalline, acidophilic, iron-negative intranuclear inclusions in the hepatic epithelial cells of a dog. The presence of these inclusions was associated with margination of chromatin, and each crystal was surrounded by a halo. He observed many similar inclusions in sections of liver from an unstated number of dogs in an additional group of 12. The animals with these inclusions were between 6 and 8½ years of age. By a variety of histochemical methods, he demonstrated that the crystals were consistently iron-free and nonlipochromic, and reacted as erythrocytes with the staining methods employed. The crystals remained intact in tissues preserved in a wide variety of fixatives, but could be dissolved in concentrated sulfuric acid. He expressed doubt as to their identification as true hemoglobin crystals. He also reported similar inclusions in the renal tubular epithelium of a dog.

Cowdry and Scott,<sup>4</sup> in 1930, described intranuclear inclusion bodies in hepatic epithelial and endothelial cells of dogs. Their description is compatible with the inclusions of canine hepatitis. They insisted that their inclusions were "different and distinct" from the intranuclear

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crystals described by Szymonowicz.<sup>2</sup> Acidophilic, crystalline intranuclear inclusions occurred in the hepatic epithelial cells of 22 per cent of the dogs which they examined. The tissues had been fixed in Kaiserling's fluid, a fixative which they regarded as unfavorable for microchemical analyses.

Nicolau and Kopciowska,<sup>5</sup> in 1936, reported acidophilic, crystalline intranuclear inclusions in the livers of 27 of 44 dogs. They also examined the kidneys from 27 dogs with inclusions in the liver and found similar bodies in the kidneys of 12. These inclusions were nonbirefringent and were demonstrated to be iron-free on microincineration. They postulated that such crystalline inclusions were caused by a saprophytic virus. Weatherford and Trimble<sup>6</sup> have indicated that they may possibly be formed from allantoin. Bloom<sup>7</sup> has expressed the thought that they probably originate from an unknown purine base and are related to uric acid excretion.

Acidophilic, crystalline intranuclear inclusions, subsequently referred to as ACN inclusions, have continued to be observed occasionally in tissue sections of the liver and kidney of dogs.<sup>8-11</sup> They have also been observed in the hepatic cells of wolves, foxes and jackals.<sup>11</sup> Their true identity and significance have not been settled. We have made an effort to characterize and identify the inclusions further, and this report presents the results of our study.

#### MATERIAL

Tissues selected at necropsy of 45 dogs and submitted to the Armed Forces Institute of Pathology (AFIP) over a period of 12 months (August, 1956 to August, 1957) were screened for the presence of ACN inclusions in liver, kidney, or both. These cases were taken at random from the hundreds submitted during this period. Tissues selected at necropsy of 30 dogs and submitted to the Pathology Branch of the United States Army Medical Nutrition Laboratory (MNL) over a period of 6 months (August, 1957 to February, 1958) were screened for the same purpose. In 13 of these 125 cases, ACN inclusions were demonstrated in liver, kidney, or both, in sections cut from formalin-fixed tissues and embedded in paraffin (Table I). None of the sections from the 13 dogs exhibited intranuclear inclusions characteristic of canine hepatitis. In cases 6, 7, 8, 10, 11, 12 and 13, ACN inclusions were also demonstrated in paraffin-embedded sections of liver and kidney fixed in absolute alcohol. They were present in both the liver and kidney in 9 cases, in the liver only in 3, and in the kidney only in 1.

TABLE I  
*Acidophilic, Crystalline Intracellular Inclusions in Dogs*

| Case no. | Accession no. | Breed, sex and age (yrs.) | Diagnoses  | ACN inclusions in Liver | ACN inclusions in epithelial cells Kidney |
|----------|---------------|---------------------------|--|-------------------------|---|
| 1        | AFIP* 783909  | German shepherd, M, 8     | Chronic focal interstitial nephritis; hemorrhage in malpighian corpuscles.   | +                       | +   |
| 2        | AFIP 744510   | Spitz, M, 6               | Pancreatic atrophy; toxic hepatitis.   | +                       | +   |
| 3        | AFIP 817665   | Boxer, M, 10              | Hemangioma, spleen; chronic interstitial hepatitis; periportal cirrhosis (moderate).   | +                       | +   |
| 4        | AFIP 772915   | Boston terrier, M, 11     | Adenocarcinoma, thyroid; malignant melanoma, with metastasis; lymphomatous enlargement, spleen; chronic passive hyperemia, liver; cystic dilatation, tubules of renal cortex; retained left testis; Sertoli cell tumor, right testis; hypertrophy of adrenals; chronic suppurative cystic hyperplasia, prostate; cortical cataract, right eye. | +                       | +   |
| 5        | MNL† 442      | German shepherd, F, 7     | Leiomyosarcoma, urinary bladder.   | +                       | +   |
| 6        | MNL 360       | Mixed breed, M, 2         | Interstitial nephritis; hemosiderosis of spleen.   | +                       | +   |
| 7        | MNL 205       | Cocker spaniel, F, 2      | Renal congestion, surgically induced.  | +                       | +   |
| 8        | MNL 405       | Pekingese, M, 2           | No significant lesions at necropsy performed after surgical level of anesthesia was maintained for 6 hours.  | +                       | +   |
| 9        | MNL 491       | German shepherd, M, 5     | Acinar atrophy, pancreas; hemosiderosis, spleen, liver, mesenteric nodes.  | +                       | +   |
| 10†      | MNL 199       | Fox terrier, M, 2         | No significant lesions at necropsy after experimental surgery.   | +                       | o   |
| 11†      | MNL 267       | Greyhound, M, 2           | No significant lesions at anatomic dissection.   | +                       | o   |
| 12       | MNL 407       | Cocker spaniel, F, 2      | No significant lesions at necropsy performed after surgical level of anesthesia was maintained for 4 hours.  | +                       | o   |
| 13†      | MNL 204       | Chow, M, 1½               | No significant lesions at necropsy after experimental surgery.   | o                       | +   |

\* AFIP = Armed Forces Institute of Pathology, Washington, D.C.

† MNL = Medical Nutrition Laboratory, Denver, Colo.

‡ Liver and kidney were the only tissues submitted to the pathology laboratory.

## METHODS

Serial sections, 7 to 8  $\mu$  in thickness, were prepared from the paraffin-embedded tissues in each case. One section of every selected tissue was deparaffined, mounted in glycerol, and examined by polarization and fluorescent microscopy, using a Philips CS 150 mercury vapor source; 6 mm. UG 1 transmission and 2.5 mm. Euphos absorption filters; or 8 mm. BG 12 transmission and 2.5 mm. OG 1 absorption filters.

Deparaffined serial sections of the selected tissues from each case were treated by a wide variety of techniques:

1. For the demonstration of cell structure: Harris' hematoxylin and aqueous eosin<sup>12</sup> and Mallory's alcoholic hyalin stain.<sup>18</sup>
2. For the demonstration of minerals: Von Kóssa's silver nitrate and Langeron's alizarin red S stains,<sup>13</sup> Gomori's iron reaction,<sup>14</sup> Macallum's stain for masked iron,<sup>15</sup> and microincineration in a muffle furnace for 30 minutes at 600° C.
3. For the demonstration of fats and lipids: Staining with osmium tetroxide,<sup>16</sup> oil red O for 24 hours,<sup>12</sup> Sudan IV,<sup>13</sup> Ziehl-Neelsen acid-fast<sup>12</sup> and Weil's myelin stains.<sup>12</sup>
4. For the demonstration of mucin, glycogen, mucopolysaccharides, polysaccharides, glycoproteins and glycolipids: The Alcian blue (short method), Mayer's mucicarmine, and periodic acid-Schiff (PAS) leukofuchsin reactions.<sup>12</sup>
5. For the demonstration of deoxyribonucleic and ribonucleic acid and nuclear proteins: The nuclear Feulgen reaction,<sup>17</sup> deoxyribonuclease and ribonuclease (supplied by General Biochemicals, Inc.) digestion,<sup>13</sup> and the ferrocyanide reaction of Hartig-Zacharias.<sup>14</sup>
6. For the demonstration of hemoglobin and its derivatives: The Ralph hemoglobin stain<sup>12</sup> and the Gmelin test.<sup>13</sup>

Tissue sections containing ACN inclusions from each case were stained with Mallory's alcoholic hyalin stain and examined microscopically.

Twelve sets of deparaffined sections from each case were subjected to the action of a variety of inorganic and organic reagents. The slides from each set were stained by Mallory's alcoholic hyalin method after treatment with the following agents at 37° C. (with the exception of boiling methanol-chloroform)<sup>18</sup>:

1. Acids: Five per cent nitric acid for one hour; saturated alcoholic picric acid for 15 minutes.
2. Organic solvents: Boiling methanol-chloroform 1:1 for 8 hours; a solution of 95 ml. of absolute ethyl alcohol and 5 ml. of 28 per cent

ammonium hydroxide for 10 minutes; ether and absolute ethyl alcohol, 1:1 for one hour, and concentrated nitric acid, 5 per cent by volume, in ether and absolute ethyl alcohol, 1:1 for one hour.

3. Oxidizing agents: Three per cent aqueous hydrogen peroxide for 30 minutes.

4. Miscellaneous agents: One per cent potassium hydroxide in 80 per cent ethyl alcohol for 10 minutes; N/10 potassium hydroxide in 75 per cent ethyl alcohol for 3 hours.

Unstained sections from each case were flooded with the following reagents and observed under a cover glass for the period of time indicated: (a) concentrated nitric acid for one hour; (b) saturated aqueous potassium hydroxide for 10 minutes.

Fresh blood from clinically normal dogs was treated by Reichert's method<sup>19</sup> and also by the saponin method<sup>20</sup> for the crystallization of oxyhemoglobin. Samples of blood were also treated by Teichmann's method<sup>19</sup> for the crystallization of hemin. Oxyhemoglobin and hemin crystals were examined by polarization and fluorescent microscopy.

#### RESULTS

In unstained, deparaffined sections of alcohol and buffered neutral formalin-fixed tissues from the 13 cases studied, the ACN inclusions had no discernible intrinsic color and were not birefringent when examined with polarized light. In glycerol-mounted sections they were not fluorescent.

In sections stained with Harris' hematoxylin and aqueous eosin or Mallory's alcoholic hyalin stains, the inclusions were usually seen as rectangular or square, or occasionally pentagonal or hexagonal, extremely acidophilic forms. They were usually surrounded by a clear halo, particularly in crosscut sections, in which they appeared as square or hexagonal forms, with plane surfaces and angular corners (Figs. 1, 3, 8 and 9). When observed in sections cut through the long axis, the halo was less apparent, and in most instances stretching of the nuclear membrane was conspicuous. At times the nucleus was stretched to several times its usual diameter, but rupture of the membrane was never observed. The volume of the cytoplasm of affected cells was not significantly increased (Figs. 2, 4 to 7, and 10). In either crosscut or longitudinal sections, the inclusions were associated with margination of chromatin and marginal displacement of the nucleolus (Figs. 1 to 4, 8 and 10). Chromatin deposition on the surface of inclusions was not observed. Occasionally 2 or 3 inclusions occurred within the same nucleus (Fig. 9).



Although the inclusions varied in size within a given section and between cases, those in the liver were more uniform than those in the kidney. The largest inclusion observed in the kidneys was invariably several times the size of the largest inclusion in the liver. The size of the liver inclusions in the 13 cases ranged from 10 to 14  $\mu$  in length, 3.5 to 4.1  $\mu$  in width and 3.0 to 4.0  $\mu$  in depth (Figs. 2, 3, 5 and 8). In the kidney, the length of the crystalline inclusions ranged from 7.2 to 32.0  $\mu$ , the width from 3.0 to 5.4  $\mu$ , and the depth from 3.7 to 4.8  $\mu$ . Although a few large inclusions were observed in each of the 10 kidneys in which inclusions were seen, most of them were of about the same size of those in the liver (Figs. 1, 4, 6, 7 and 10).

The results obtained with the other techniques employed were uniformly identical and will be described collectively. Negative results were obtained with each of the following staining procedures or histochemical reactions: von Kóssa's silver nitrate, Langeron's alizarin red S, Gomori's iron reaction, Macallum's masked iron, osmium tetroxide, oil red O, Sudan IV, Ziehl-Neelsen acid fast, Weil's myelin, Alcian blue, Mayer's mucicarmine, periodic acid-Schiff leukofuchsin, nuclear Feulgen, deoxyribonuclease and ribonuclease digestion, Ralph's hemoglobin, the ferrocyanide reaction of Hartig-Zacharias, and the Gmelin test. In spodograms the inclusions did not leave mineral ash.

The inclusions were not soluble in any of the inorganic and organic agents to which the tissue sections were subjected. Hydrogen peroxide was the only agent which affected the affinity of the inclusions for Mallory's alcoholic hyalin stain. Inclusions treated with hydrogen peroxide stained pink rather than the characteristic intense red.

Regardless of the method of preparation employed, oxyhemoglobin and hemin crystals from canine blood were birefringent and non-fluorescent. Hemin crystals were generally larger than ACN inclusions in tissue sections and were rhomboid rather than rectangular.

#### DISCUSSION

We considered the possibility that ACN inclusions might be artifacts incident to fixation. However, since we observed them in paraffin-embedded tissue fixed in buffered or unbuffered neutral 10 per cent formalin or absolute alcohol, and Cowdry and Scott<sup>21</sup> described them in tissue fixed in Kaiserling's fluid, we believe that they cannot be fixation artifacts.

Although repeated reference has been made to the probable identity of ACN inclusions as crystalline hemoglobin or hemoglobin derivatives,<sup>1-4,9</sup> we could not demonstrate by Gomori's iron, Macallum's masked iron or microincineration techniques that they contained

hemoglobin or any detectable iron. That organic or inorganic iron could not be demonstrated by the Gomori and Macallum reactions would not in itself be conclusive proof that the inclusions did not contain the metal.<sup>22</sup> Microincineration, however, is known to be a very sensitive method for demonstration of iron in tissue<sup>22</sup> and in individual red blood cells. In spodograms, iron leaves a characteristic brownish-red ash, but the ACN inclusions left no such ash and, in fact, were totally consumed by this method. Therefore, we believe that the inclusions do not contain iron. Evidence that iron was absent, coupled with the fact that crystals of canine oxyhemoglobin and hemin are birefringent and the inclusions were not birefringent constituted sufficient proof that ACN inclusions were not composed of hemoglobin or any of its iron-containing protoporphyrin precursors or derivatives.<sup>23</sup> These observations reinforce the doubts expressed by Brandts<sup>3</sup> that the inclusions were hemoglobin crystals and substantiate the observation of Nicolau and Kopciowska<sup>5</sup> that they did not contain iron.

It has been reported that polymerized or oxidized unsaturated lipids may be present in paraffin sections under various conditions.<sup>13</sup> In such situations and at adequate temperatures, these lipids may exist as crystals and may be birefringent. Certain of them exhibit fluorescence and positive reactions to lipochromic staining and the PAS leukofuchsin techniques. The ACN inclusions did not exhibit any of these physical or lipochromic properties and were not stainable by the PAS leukofuchsin reaction, and thus could not be identified as lipids. That the ACN inclusions are composed in part or wholly of substances such as glycogen, mucin, mucopolysaccharides, polysaccharides, glycoproteins or glycolipids was disproved by their consistently negative reactions to histochemical methods which are generally held to be adequate for the demonstration of these substances.

The possible leads which we have discussed so far are admittedly empirical, but were suggested by some of the earlier reports in the literature. Our main effort was directed toward determining the possible role of organic material normally present within the cytoplasm and nucleus of animal cells in the formation of abnormal structures, in this case the ACN inclusion.

Cholesterol is a substance that is universally present in animal cells.<sup>24</sup> The possibility that the inclusions were composed of cholesterol was investigated. They were nonbirefringent and insoluble in ether, xylene or strong alcohol, whereas cholesterol is birefringent<sup>13</sup> and soluble in these fluids. These differences eliminated cholesterol as the basic component of ACN inclusions.

The first of the nuclear components to be evaluated was deoxyribo-

nucleic acid. It is normally basophilic and gives the nuclear Feulgen reaction.<sup>18,14,25</sup> The negative Feulgen reaction exhibited by the ACN inclusions indicated that they did not contain deoxyribonucleic acid. Because it was believed that the crystalline structure of the inclusions might have interfered with the reaction, however, deoxyribonuclease digestion of the ACN inclusions was attempted. Since the action of most proteolytic enzymes, and enzymes in general, is relatively specific,<sup>26</sup> this procedure constituted a definitive test for the histochemical identification of deoxyribonucleic acid. The ACN inclusions were unaffected by such digestive procedures; therefore it was concluded that they were not composed of deoxyribonucleic acid.

We also postulated that the inclusions might be composed partly or wholly of ribonucleic acid, derived either from the cytoplasm or the ribose nucleotides present in most nucleoli.<sup>27</sup> Ribonucleic acid is basophilic in its staining properties, and digestion by ribonuclease is accepted as the definitive test for its histochemical identification.<sup>18</sup> The strong acidophilia and resistance of the inclusions to ribonuclease digestion indicated that these inclusions did not contain demonstrable ribonucleic acid. It was assumed that they were not composed of pyrimidines or purines since they were insoluble in dilute aqueous alkali or mineral acid.<sup>28</sup> Because of their insolubility in ethyl alcohol, the inclusions were not thought to be composed of purine end products such as allantoin.

The nuclear components to which the ACN inclusions might most logically be related were, in our opinion, the nuclear proteins. Normal nuclear components, such as the chromosomes and nucleoli, are partially composed of varying quantities of nucleoproteins; viruses and rickettsias also contain nucleoproteins.<sup>27</sup> Therefore, in the attempt to characterize the ACN inclusions, the possibility that they were viral inclusions had to be considered.

Round, acidophilic, intranuclear inclusions of Cowdry's types A and B<sup>29</sup> have been reported in the renal tubular epithelium of man<sup>9,21</sup> and a number of birds<sup>30</sup> and animals.<sup>21,30-32</sup> Usually these inclusions were either demonstrated or thought to be associated with a virus. In no instance were they described or depicted as crystalline, or their plane surfaces as rectangular or square with angular corners.

We did not conduct tests to determine whether the inoculation of fresh tissue homogenates from animals with ACN inclusions would produce analagous lesions in suitable laboratory animals. We depended on histochemical procedures to demonstrate whether the inclusions shared certain known chemical characteristics of viral inclusion bodies.

It has been reported that the cytoplasmic inclusion bodies of various viruses, when subjected to microincineration, leave a mineral ash, whereas nuclear viral inclusions leave little if any mineral ash and the nucleoli of affected cells generally contain abundant mineral.<sup>33</sup> By microincineration we were not able to demonstrate any mineral ash in the residue of the ACN inclusions, and must conclude that they possess this characteristic in common with viral intranuclear inclusions.

Although only a few viruses have been investigated histochemically, some of the large viruses and their inclusions have been reported to give a positive Feulgen reaction.<sup>27,34,35</sup> Variable Feulgen reactions, evidently associated with the age of the inclusion body, have been reported for the Negri body<sup>27,36</sup> and the inclusion of herpes simplex.<sup>35</sup> In regard to virus reproduction, Caspersson stated: "The organization of the organelles for protein production, which we have just described for simpler organisms, shows that the same basic outlines are found here, too [virus reproduction]. Ribose nucleotides take part in the reproduction of even the simplest viruses consisting of only one protein group. The higher types of viruses, which we must assume contain several different kinds of protein that are simultaneously multiplied and distributed to the daughter particles, have a primitive mechanism that performs at least some of the functions of the chromosomes in higher forms. They contain the otherwise chromosome-specific nucleotide, ribodessose nucleic acid."<sup>27</sup> If one accepts this thesis, it would be logical to assume that if the inclusion body produced by or associated with a given virus contains live virus, ribonucleic acid should be demonstrable in the inclusions of simple viruses and deoxyribonucleic acid in the inclusions of higher viruses. Although the evidence that ACN inclusions are not related to a virus is inconclusive, the absence of ribonucleic and deoxyribonucleic acid in the ACN inclusions lessened the probability that they were viral inclusions.

There are also certain physical properties of viral inclusions which are characteristic. Cowdry<sup>29</sup> described intranuclear inclusions as being of two types, A and B. Type A inclusions contain little or no mineral matter or deoxyribonucleic acid. They are amorphous, may be condensed into rounded masses, and are associated with a total nuclear reaction which proceeds to complete necrosis. Type B inclusions occur in nuclear foci which may be large or small and of hyaline appearance. The chromatin does not marginate and may accumulate on the inclusion. Furthermore, nuclear degeneration is seldom complete, and a tissue reaction is seldom observed. Cowdry and associates observed ACN inclusions in the livers of dogs<sup>4,30</sup> and noted that they bore some re-

semblance to type B inclusions in that the chromatin appeared to be pushed aside rather than margined, and degeneration was only partial. They differed from type B inclusions in that they were distinctly crystalline. Cowdry and Scott<sup>4</sup> accurately described the ACN inclusions observed in the livers of a large number of dogs and drew attention to their large size, their length (which ranged from 10 to 25  $\mu$ ), and their distinctive shape when cut through the long axis. Only when they were cut in cross section did the ACN inclusions sometimes suggest inclusions caused by a filtrable virus. They were of the opinion that ACN inclusions, because of their physical properties and because of the absence of nuclear necrosis, were not similar to viral inclusions.

The ACN inclusions exhibited several features which are different from those of known viral inclusions: (1) They were crystalline, with plane surfaces and angular corners. (2) They varied widely in size, especially in length (7.2 to 32.0  $\mu$ ), which greatly exceeded that of most known viral inclusion bodies. (3) Although they stretched the nuclear membrane to great lengths and crowded the nucleolus and chromatin toward the nuclear membrane, chromatin did not accumulate on the surfaces of the inclusions, and nuclear necrosis was not evident. (4) They did not contain demonstrable deoxyribonucleic acid or ribonucleic acid. (5) In the cases observed, there was no disease to which they could be related. Although these characteristics do not conclusively distinguish the ACN inclusions from viral inclusions, they indicate that ACN inclusions are certainly different from known viral intranuclear inclusions and raise doubts as to their possible identity as such.

The possible role of the proteins produced by the nucleolus-associated chromatin in cells at the time of profuse production of protein cannot be overlooked as a possible source of ACN inclusions. Caspersen has defined nucleoli as "... dense, rounded, as a rule optically homogeneous, endonuclear bodies consisting of proteins in high concentrations, rich in diamino acids and associated with the cytoplasmic protein formation."<sup>27</sup> As demonstrated by microspectrophotometry, at the time when formation of proteins is increased at the site of the nucleolus-associated chromatin, there is a concomitant increase in proteins in the cytoplasm.<sup>27</sup> The concentration of proteins, at the time of intensified production, is greatest at the nucleolus; there is diffusion of these substances from the nucleolus to the nuclear membrane, and from nuclear membrane to the cell membrane, where the concentration is lowest.<sup>27</sup> It may be debated whether protein diffuses across the nuclear membrane or protein formation proceeds at differing intensities

throughout the cell. Assuming that protein formed at the nucleolus diffuses outward, it follows that ACN inclusions may represent crystallization of protein within the nucleus, associated with lowered permeability of the nuclear membrane.

That the ACN inclusions are negative to the ferrocyanide reaction of Hartig-Zacharias does not constitute proof that they do not contain protein.<sup>14</sup> Most methods employed for the histochemical identification of protein represent application of gross biochemical methods, and are of doubtful accuracy or usefulness.<sup>14,37</sup> Several factors add credence to the theory that the inclusions represent crystallization of protein: (a) their consistently strong acidophilia; (b) the lack of evidence of necrosis of cytoplasm or of affected nuclei; (c) the presence of small nucleoli within affected cells; (d) the evident toughness of the nuclear membrane, which in some cases was stretched to accommodate inclusions over 30  $\mu$  in length with no evidence of broken continuity; (e) the noticeable lack of increase in volume of the cytoplasm; and (f) the fact that the inclusions were composed of organic substances and left no mineral ash upon microincineration. On the other hand, if the inclusions are a result of altered metabolism of nuclear protein, why were only hepatic and renal tubular epithelial cells affected?

A possible viral cause of the ACN inclusion cannot be ruled out. Furthermore, the relation of possibly altered permeability of the nuclear membrane, which favors retention of protein intranuclearly at periods of intensive protein synthesis in the nucleus, merits further study. These two possible etiologic factors could well be interrelated. It must be emphasized that both factors are hypothetical. Conclusive results which would confirm or deny the role of a virus or altered nuclear protein metabolism in the formation of ACN inclusions were not demonstrated in this study.

On the basis of the results obtained, ACN inclusions may be characterized as nonbirefringent crystalline intranuclear inclusions composed of acidophilic organic material, which occasionally occur in epithelial cells of the liver and kidneys of dogs of either sex, ranging in age from 18 months to over 11 years. They have plane surfaces and angular corners and edges, and are usually observed in sections as square or rectangular and occasionally as hexagonal or pentagonal forms. These stretch the nuclear membrane and displace the chromatin and nucleolus to the periphery of the nucleus. Extreme variations in size are observed between individual inclusions and host cells. Nuclear degeneration is not evident in most affected cells. The inclusions are not accompanied by an inflammatory response in affected tissues.



## SUMMARY

Acidophilic, crystalline intranuclear inclusions were observed within epithelial cells of the liver and kidneys of 13 dogs. Histochemical and physical procedures carried out upon paraffin-embedded tissue sections showed that they were not composed of hemoglobin or any of its iron-containing derivatives, nor of minerals, lipids, deoxyribonucleic acid, ribonucleic acid, cholesterol, glycogen, mucin, mucopolysaccharides, polysaccharides, glycoproteins, or glycolipids. Although the inclusions exhibited many characteristics unlike those of known viral inclusions, their possible identity as such must still be considered.

The possible role of altered protein metabolism within affected nuclei was considered, and evidence which might support this theory was adduced, but the true identity and significance of the inclusions remain unknown.

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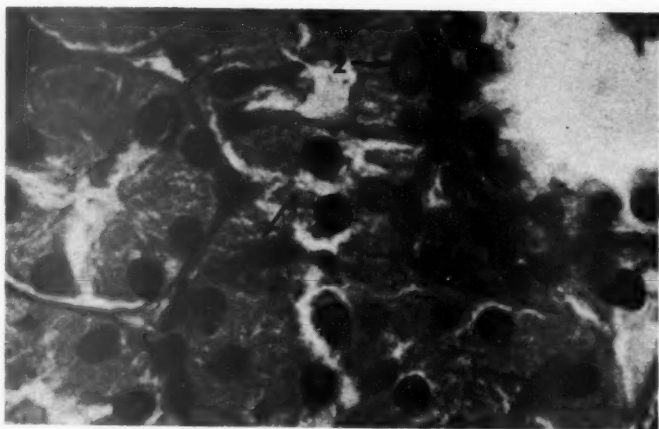
The authors appreciate the editorial assistance and criticism furnished by the late Mrs. Helen Knight Steward, Editor, and Dr. Frank Johnson, Chief of the Histochemistry Section of the Armed Forces Institute of Pathology.

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#### LEGENDS FOR FIGURES

All photomicrographs were taken by Mr. William Hummel of the Pathology Service, Fitzsimons Army Hospital, Denver, Colo.

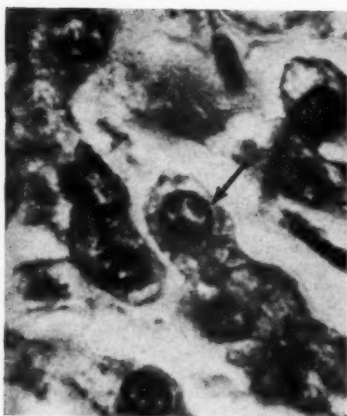
- FIG. 1 (AFIP Accession 783909). ACN inclusions in nuclei of renal tubular epithelial cells from case 1. Note the variations in size of the rectangular inclusions (1) and the displacement of the nucleoli to the nuclear membrane (2). One hexagonal inclusion is cut transversely (3). Mallory's alcoholic hyalin stain.  $\times 1,000$ .
- FIG. 2. (AFIP Accession 783909). Rectangular inclusion in the nucleus of a liver epithelial cell from case 1. Note the intact stretched nuclear membrane. Hematoxylin and eosin stain.  $\times 1,000$ .
- FIG. 3 (AFIP Accession 817655). A small ACN inclusion lying against the nuclear membrane of a liver epithelial cell from case 3. Note the angular corners of the inclusion and the marginal displacement of the chromatin and nucleolus. Mallory's alcoholic hyalin stain.  $\times 1,000$ .
- FIG. 4 (AFIP Accession 772915). A single ACN inclusion, measuring  $32 \mu$  in length, in the nucleus of a renal cortical epithelial cell from case 4. Mallory's alcoholic hyalin stain.  $\times 1,000$ .
- FIG. 5 (MNL Accession 360). A single ACN inclusion, measuring  $10 \mu$  in length, within the nucleus of a hepatic epithelial cell from case 6. Note the stretching of the nuclear membrane and marginal displacement of the nucleolus. Mallory's alcoholic hyalin stain.  $\times 1,000$ .



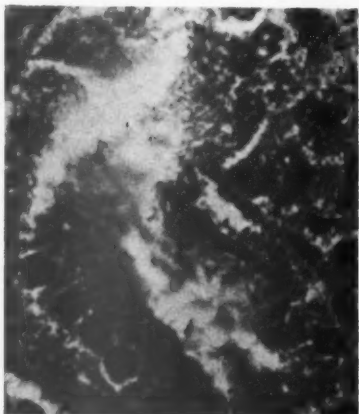
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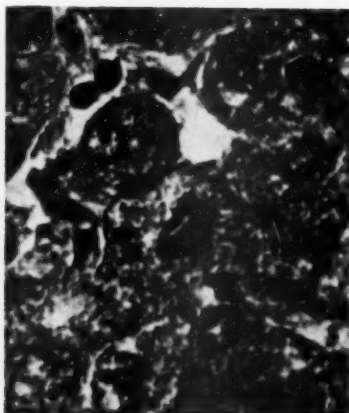
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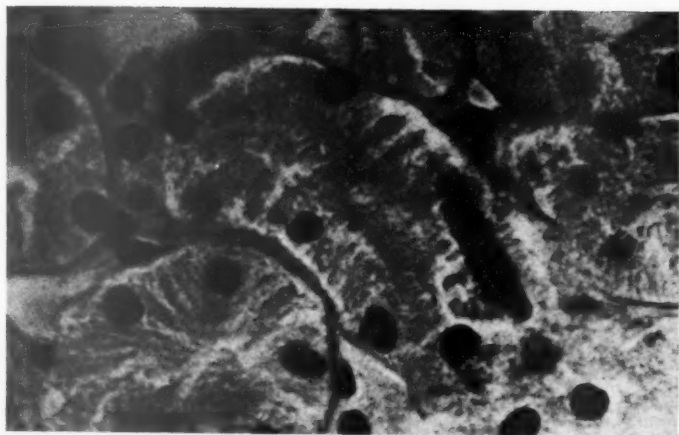
FIG. 6 (MNL Accession 360). A single inclusion, measuring  $22\ \mu$  in length, in the nucleus of a renal cortical epithelial cell, showing irregular margination of chromatin, from case 6. Mallory's alcoholic hyalin stain.  $\times 1,000$ .

FIG. 7 (MNL Accession 405). A single inclusion, measuring  $17\ \mu$  in length, in the nucleus of a renal cortical epithelial cell, showing irregular margination of chromatin, from case 8. Mallory's alcoholic hyalin stain.  $\times 1,000$ .

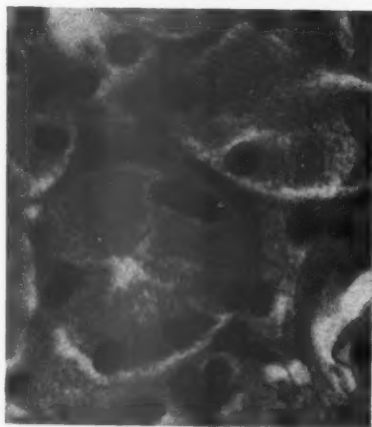
FIG. 8 (MNL Accession 491). A single hexahedral inclusion, cut transversely, in the nucleus of a liver epithelial cell from case 9. Mallory's alcoholic hyalin stain.  $\times 1,000$ .

FIG. 9 (MNL Accession 407). Three inclusions are present within the nucleus of a single liver epithelial cell from case 12. Mallory's alcoholic hyalin stain.  $\times 1,000$ .

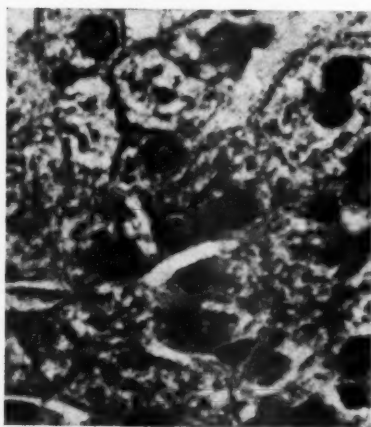
FIG. 10 (MNL Accession 204). ACN inclusions in the nuclei of epithelial cells of a renal cortical tubule from case 13. Note the marginal displacement of nucleoli and chromatin in each affected nucleus. Mallory's alcoholic hyalin stain.  $\times 1,000$ .



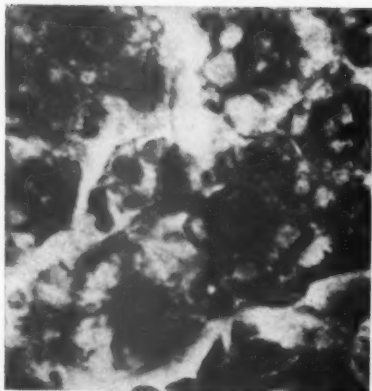
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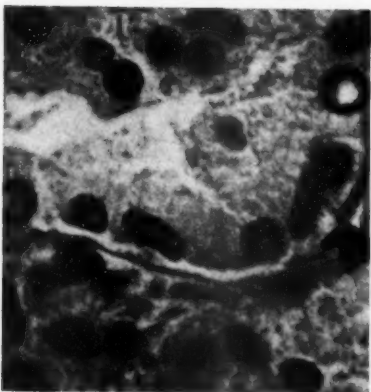
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## THE EFFECTS OF TREATMENT ON THE STERNAL MARROW\*

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Pettet, Pease and Cooper<sup>1</sup> recently have reported the incidence of specific neoplastic invasion of the sternal marrow in cases of malignant lymphoma. For some time, Gormsen, Sundberg, and others<sup>2-4</sup> have been reporting the occurrence of nonspecific granulomatous lesions in patients suffering from tuberculosis, histoplasmosis, brucellosis, sarcoidosis and infectious mononucleosis. Pease<sup>5,6</sup> has noted these nonspecific granulomas in cases of malignant lymphoma. All the studies mentioned were done on sternal marrow aspirates from the living patient.

During the course of an investigation concerning the incidence of malignant lymphomatous infiltration and nonspecific granulomas in the sternal marrow in patients with malignant lymphoma in whom necropsy was performed, consideration was given to the effects of treatment on the sternal marrow. It is the purpose of this presentation to report on these effects.

### MATERIAL AND METHODS

All patients with malignant lymphoma who had necropsy examination at the Mayo Clinic during a 5-year period from January 1, 1951, to December 31, 1955, were studied. Seventy-nine such patients provided adequate material for our purposes. In 32 a reticulum cell sarcoma was present; in 26 the lesions were those of Hodgkin's disease; in 20, lymphosarcoma; and in one, giant follicular lymphoma.

The sternal marrow taken at necropsy examination from the level of the second intercostal space and mounted in paraffin blocks was sectioned at 10 different levels. Approximately two thirds of the available tissue was used. At each level, as many consecutive sections (5  $\mu$  in thickness) were examined as could be mounted on one standard glass slide; the number of sections per slide varied from 2 to 9. These were stained with hematoxylin and eosin. From 20 to 90 sections were examined in a systematic manner in each case. The cellularity of the

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marrow and the amount of destruction of tumor and marrow elements, if any, were recorded. The cellularity was judged as normal when the ratio of cellular elements to the fatty tissue was between 1:1 and 3:1. When this ratio was greater than 3:1, the preparation was termed "hyperplastic"; when it was smaller than 1:1, "hypoplastic," and when virtually no marrow cells were seen, it was termed "aplastic."

Fifty-three patients had been treated by irradiation (roentgen rays or radioactive cobalt); 44 had received nitrogen mustard (HN<sub>2</sub>); transfusions had been administered to 45; adrenocortical steroids to 13, and triethylene melamine (TEM) to 8. Most patients had received more than one type of treatment in various combinations. One additional patient with lymphosarcoma was treated with radioactive phosphorus.

## RESULTS

### *Effect on Cellularity*

As part of the therapeutic program, radiation therapy not directed to the mediastinum was used in 25 patients; the sternal marrow in 12 of these was either hyperplastic or normal in cellularity; in the remaining 13 cases, the sternal marrow was hypoplastic or aplastic. When radiation had been directed to the mediastinum, the sternal marrow in 4 instances was normal or hyperplastic, whereas in 24 it was hypoplastic or aplastic. The final pattern in the sternal marrow in instances in which radiation therapy had been given to the mediastinum within an interval of one month prior to the time of death did not differ significantly from that in cases in which irradiation had been used for as long as one year before death.

Of the 26 patients receiving nitrogen mustard within a month prior to death, 10 had hyperplastic or normal marrow, and 16 had hypoplastic or aplastic marrow. Of the 18 patients who had received nitrogen mustard more than one month prior to death, 4 had hyperplastic or normal marrow, while 14 had hypoplastic or aplastic marrow.

Two features are worthy of emphasis: (1) Of the 20 patients receiving both nitrogen mustard and radiation therapy directed to the mediastinum at some time during the course of the disease, only one had hyperplastic marrow, and 19 had either hypoplastic or aplastic marrow. (2) Of the 15 patients who received neither radiation therapy nor chemotherapy, 12 had normal or hyperplastic marrow, and only 3 had hypoplastic or aplastic marrow.

The patient who received radioactive phosphorus had an aplastic marrow at the time of death. The effect of triethylene melamine administered on 8 occasions was difficult to evaluate because it was combined in 7 instances with both roentgen therapy and nitrogen

mustard and once with nitrogen mustard alone. Of the 8 patients receiving triethylene melamine, 7 had hypoplastic or aplastic sternal marrow, and one had hyperplastic marrow.

#### *Effect on the Occurrence of Neoplastic Infiltration*

Of the 20 patients given both nitrogen mustard and radiation therapy to the mediastinum, 5 (25 per cent) showed neoplastic infiltration of the sternal marrow; of the 59 who did not receive both types of treatment in this manner, 21 (36 per cent) had tumorous infiltration.

#### *Extent of Necrosis*

Necrosis was graded with a range of 1 to 4. If an area less than 25 per cent of that of the whole preparation (a single slide) was necrotic, necrosis was classed as grade 1; from 25 to 50 per cent, grade 2; from 50 to 75 per cent, grade 3; and more than 75 per cent, grade 4. It was usually not possible to differentiate necrotic tumor tissue from necrotic marrow tissue. In the 26 patients who received nitrogen mustard within one month prior to death, 8 (31 per cent) had necrosis, grade 3 or 4. Only one of 18 patients (6 per cent) showed grade 3 necrosis when longer than one month had elapsed between the administration of nitrogen mustard and the time of death. There was no significant difference in the amount of necrosis between those receiving radiation therapy to the mediastinum and those receiving it elsewhere; in the latter category, however, 58 per cent had received nitrogen mustard in addition.

#### *Comparison Between Aspiration and Necropsy Tissue*

Aspiration of bone marrow was carried out during life in 19 of the 79 patients. In 9 some form of therapy (roentgen therapy or cobalt irradiation, nitrogen mustard or radioactive phosphorus) had been given between the time of sternal aspiration and death. The observations are summarized in Table I. In most instances, nitrogen mustard given within 2 months of death had caused some decrease in the normal cellularity of the preparations, and in 3 cases, appreciable areas of necrosis (Figs. 1 and 2).

#### *Incidence of Neoplastic Infiltration*

In 26 of the 79 patients (33 per cent), there was specific infiltration of the sternal marrow by neoplastic tissue as seen by serial sectioning (Fig. 3). The invasion was focal in 23 instances and diffuse in 3. Such tumor invasion was seen in 20 per cent of the cases of reticulum cell sarcoma, in 38 per cent of the patients with Hodgkin's disease, and in

45 per cent of those with lymphosarcoma. In only one of the 26 patients was the marrow the only demonstrable site affected; in the other 25, the lymphomatous process was generalized.

TABLE I  
*Effect of Treatment Between the Time of Marrow Aspiration and Death*

| Lesion                 | Treatment                | Days from treatment to death | Sternal marrow |                               |
|------------------------|--------------------------|------------------------------|----------------|-------------------------------|
|                        |                          |                              | Aspiration     | Necropsy specimen             |
| Hodgkin's disease      | HN <sub>2</sub> *        | 21                           | Normal         | Hypoplasia; necrosis, grade 4 |
| Hodgkin's disease      | HN <sub>2</sub>          | 60                           | Normal         | Normal                        |
| Hodgkin's disease      | HN <sub>2</sub>          | 10                           | Hyperplasia    | Hypoplasia; fibrosis, grade 4 |
| Hodgkin's disease      | Co <sup>60</sup>         | 15                           | Hyperplasia    | Hypoplasia; necrosis, grade 1 |
| Lymphosarcoma          | P <sup>32</sup>          | 30                           | Hyperplasia    | Aplasia                       |
| Lymphosarcoma          | HN <sub>2</sub><br>X-ray | 45                           | Hyperplasia    | Hypoplasia                    |
| Reticulum cell sarcoma | HN <sub>2</sub>          | 14                           | Hypoplasia     | Hypoplasia; necrosis, grade 3 |
| Reticulum cell sarcoma | HN <sub>2</sub>          | 60                           | Normal         | Normal                        |
| Reticulum cell sarcoma | HN <sub>2</sub>          | 10                           | Hypoplasia     | Aplasia; necrosis, grade 4    |

\* HN<sub>2</sub> = Nitrogen mustard.

A careful search was made for nonspecific granulomas by scanning the cross sections in a systematic manner. No lesion satisfying the criteria as laid down by Pease<sup>6</sup> was found.

#### DISCUSSION

These results indicate that radiation therapy must be aimed directly at the mediastinum in order to produce maximal destructive effects on the cellularity of the sternal marrow. Nitrogen mustard, on the other hand, rather consistently provokes destructive alterations in sternal marrow when administered in customary intravenous palliative dosages. The greatest destruction of marrow tissue was seen when both of these methods were used in the same patient. The arbitrarily chosen interval of one month after therapy did not seem to be important in determining the final pattern in the marrow, except perhaps in the necrosis produced by nitrogen mustard. It seemed that treatment in itself had little influence on the incidence (33 per cent) of specific neoplastic infiltration of the sternal marrow at necropsy. Radiation therapy directed to the mediastinum and nitrogen mustard were effective in destroying normal sternal marrow tissue but not all lymphomatous

tissue. We have no good explanation to offer for our failure to discover nonspecific granulomas in necropsy specimens, when these have been seen in the bone marrow obtained by aspiration during life in approximately 8 per cent of cases.<sup>6</sup> However, none of the patients in whom Pease<sup>6</sup> demonstrated these granulomas during life were included in this clinicopathologic study.

The results have been presented for malignant lymphomas as a group; no significant differences in the phenomena could be demonstrated among any of the 4 main groups of malignant lymphoma.

#### SUMMARY

The tissues from 79 patients with malignant lymphoma upon whom necropsy was performed at the Mayo Clinic during a 5-year period, 1951 through 1955, were reviewed. The specimen of sternal marrow taken at the time of necropsy examination was serially sectioned. Radiation therapy directed toward the mediastinum, as well as the intravenous administration of nitrogen mustard, seemed to be directly implicated in the causation of hypoplasia, aplasia, and necrosis of the sternal marrow. Radiation therapy not directed toward the mediastinum did not have such a destructive effect on the normal cellularity of sternal marrow. When nitrogen mustard was given to patients also receiving direct mediastinal irradiation, the destruction of marrow tissue was rather extensive. In 26 of the 79 patients (33 per cent), there was neoplastic tissue in the sternal marrow at necropsy. We were unable to find any nonspecific granulomas in the marrow.

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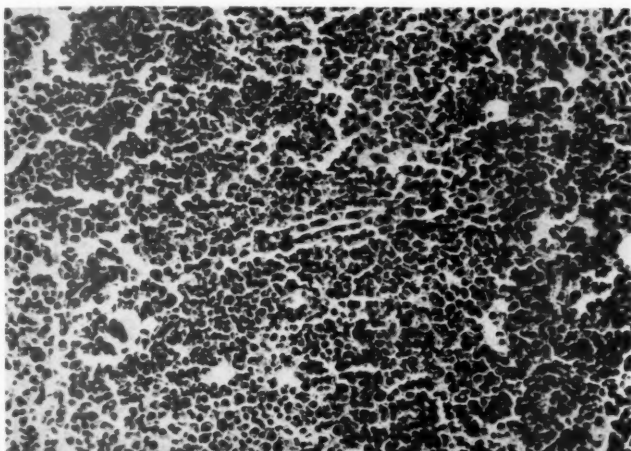
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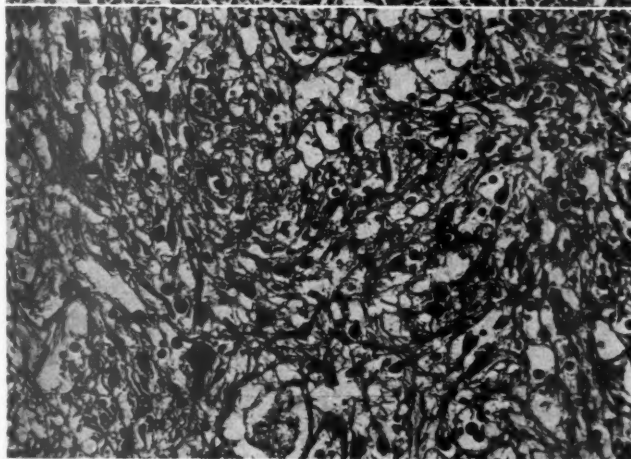
[ Illustrations follow ]

## LEGENDS FOR FIGURES

- FIG. 1. Hyperplastic sternal marrow obtained by aspiration. Hematoxylin and eosin stain.  $\times 210$ .
- FIG. 2. Marrow from same patient shown in Figure 1. Specimen obtained at necropsy 10 days after nitrogen mustard had been administered, showing zones of hypoplasia and fibrosis. Hematoxylin and eosin stain.  $\times 210$ .
- FIG. 3. Neoplastic infiltration of the sternal marrow in Hodgkin's disease with Reed-Sternberg giant cells. Hematoxylin and eosin stain.  $\times 400$ .



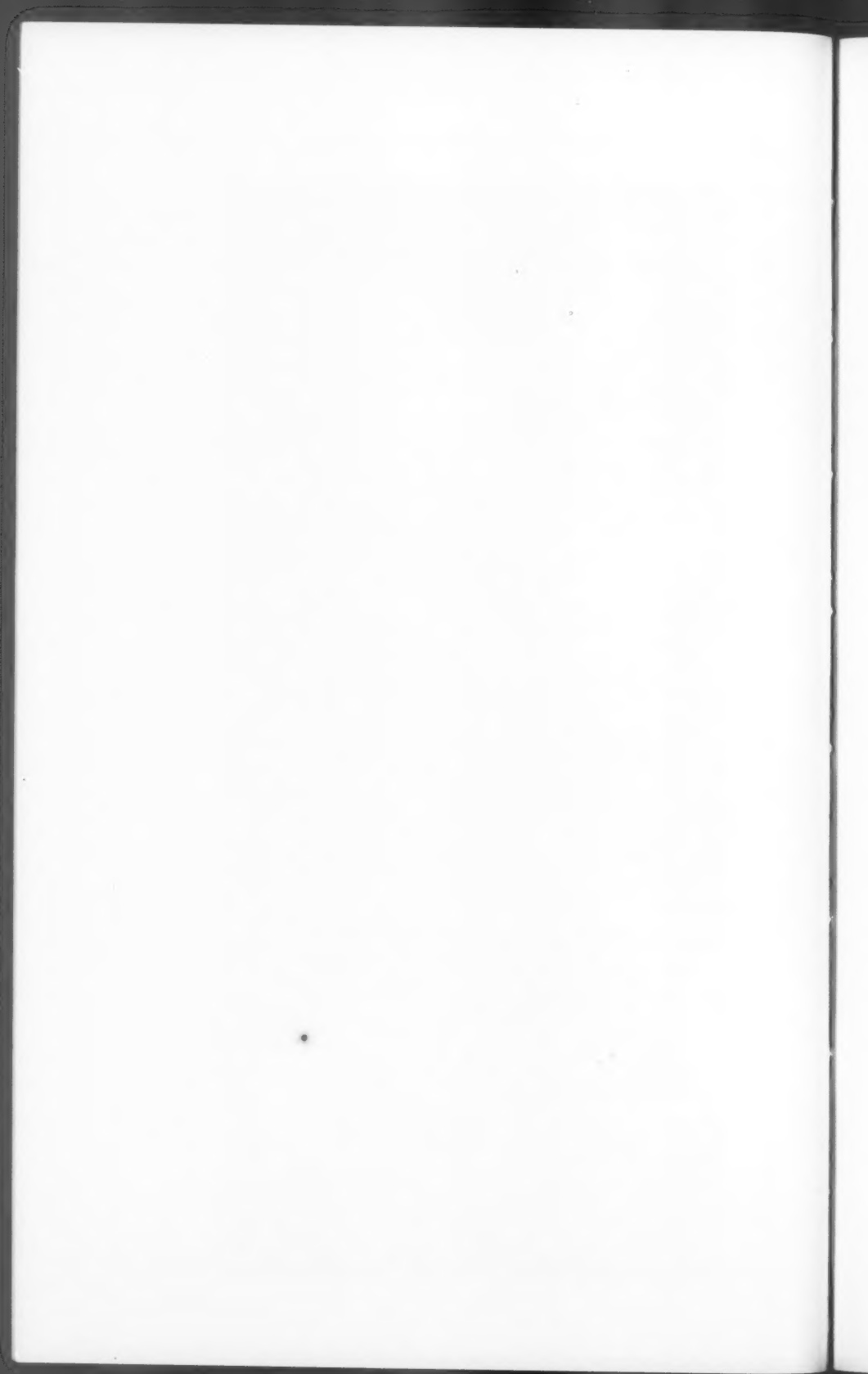
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## REGRESSION OF TRAUMATIC CUTANEOUS AND AURAL LIPID DEPOSITS IN CHOLESTEROL FED RABBITS\*

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A previous study<sup>1</sup> has indicated that lipid deposits in cutaneous xanthomas of man are more readily resorbed or converted into fibrous tissue than are lipid deposits in arterial atheromatous plaques. The present report furnishes evidence that this is also true of extra-arterial lipid deposits in cholesterol-fed rabbits.

In rabbits fed cholesterol, the blood and liver content of cholesterol increases long before deposits of this substance can be demonstrated in the arterial intima. In fact there is usually a latent period of 4 to 5 weeks before vascular deposits become visible. It has been suggested that during this period the blood and tissues are "saturated" with cholesterol. Kellner and Chang<sup>2</sup> have shown that the cholesterol content of lymph is also increased. In spite of very high blood levels, cholesterol-fed rabbits ordinarily do not develop widespread xanthomatous skin eruptions during a 10-week feeding period. Nevertheless, an increased content of cholesterol is found not only in liver but in such sites as the spleen, bone marrow, adrenal cortex, and cornea. Occasionally there are focal xanthomatous deposits in the kidney, lung or heart as well. Skin lesions of this type are found chiefly at pressure points, as in the foot pads. Incidental inflammatory lesions in hypercholesterolemic rabbits are likely to harbor large amounts of cholesterol within phagocytic cells. Traumatized areas, such as points of venipuncture,<sup>3</sup> also are, on occasion, sites of xanthoma formation. These observations suggest that there may be some correlation between the deposition of cholesterol in arteries and in extra-arterial sites.

### EXPERIMENTS AND RESULTS

#### *Resorption of Intradermally Injected Hypercholesterolemic Rabbit Serums in Hypercholesterolemic Rabbits*

If the increased content of cholesterol in tissue fluids is responsible for the tendency to develop extra-arterial lipid accumulations at points of injury, it is reasonable to suspect that the ability to remove paren-

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terally injected cholesterol would be impaired in hypercholesterolemic rabbits. The serums of a group of rabbits, made hypercholesterolemic, as previously described,<sup>4</sup> by feeding 1 gm. of cholesterol daily for 10 weeks, were collected. The serums of 6 of these, in which the total serum cholesterol varied from 1,500 to 2,000 mg. per hundred cc. (Schoenheimer and Sperry's method), were injected intradermally into 5 sites in the abdominal region in 1 cc. amounts. Each of 5 hypercholesterolemic rabbits received its own serum, that of 2 other hypercholesterolemic rabbits, and serums from 2 normal rabbits. In addition, 4 normal rabbits received intradermal injections of 1 cc. of hypercholesterolemic serum and 1 cc. of normal rabbit serum at different sites. The wheals at all inoculated sites disappeared with equal rapidity. Within one hour the site of injection was barely visible.

In order to increase the amount of cholesterol introduced into a small area, 6 hourly, 1 cc. injections of the same serum were made into the same sites. It was estimated that from 90 to 120 mg. of cholesterol was thus injected at all sites receiving hypercholesterolemic serum. At the end of the sixth hour there was no evidence of any residue of the inoculums. The skin at the sites of injections was excised and fixed in 10 per cent formalin. In Sudan IV stained, frozen sections there was no evidence of abnormal lipid accumulations. The experiment was repeated in a second group of 4 hypercholesterolemic rabbits, adding 0.1 cc. of adrenalin to each cubic centimeter of serum in order to retard resorption. No evidence of lipid substance, however, was found at the sites of multiple injections.

These observations indicated that hypercholesterolemic rabbits were able to remove considerable amounts of injected serum cholesterol from interstitial tissue and, under the conditions of the experiment, did so as readily as rabbits with normal blood cholesterol levels.

#### *Xanthogranulomatous Aural Lesions*

Continued tissue injury may be required to cause the local accumulation of cholesterol in hypercholesterolemic rabbits. Many rabbits develop cutaneous and subcutaneous thickenings about the margins of ordinary aluminum ear tags (Aluminum Marker Works, Beaver Falls, Pennsylvania). To some extent the frequency of such swellings depends on how firmly the metal disc is pressed against the skin. In our experience such lesions were found at the edges of 50 to 65 per cent of all rabbits several weeks after the tags had been inserted. Histologic examination of the lesions revealed a nonspecific chronic inflammatory reaction in which little or no stainable lipid was present.

Such lesions were likely to reach a fixed size or to progress very slowly.

In cholesterol-fed rabbits, however, after about 5 weeks, at a time when lipid deposits first become grossly visible in arteries, the aural swellings often began to enlarge and become nodular. The nodules occasionally completely engulfed the metal ear tag and formed a disc-like, hard mass measuring up to 2 cm. in diameter by the tenth week of cholesterol feeding. They did not appear, however, unless there was some indication of local injury prior to the development of hypercholesterolemia. Both the outer and inner ear skin surfaces might show some enlargements, but they were apt to be more striking on the inner surface of the ear. Since the tags are attached to the ears of all rabbits in our laboratory routinely, many aural lesions in rabbits used as controls in other experiments were available.

Histologic examination revealed an extraordinary amount of extra- and intracellular lipid substance in the granulation tissue of the lesions. The crystals appeared as flat plates and were soluble in fat solvents, doubly refractile in polarized light and did not stain with Sudan IV. They were, therefore, considered to be composed of free cholesterol. On the other hand, a large amount of sudanophilic substance was found in large mononuclear phagocytes. In some instances, free masses of cholesterol and keratin were lodged between the flat surfaces of the ear tag and the overhanging and enclosing edges of the nodule.

Since these xanthogranulomatous lesions first became evident at about the time when arterial lipid deposits are known to appear, it was thought that some correlation might exist between the size of the ear nodules and the extent of arterial lipid deposition. If this were true, the development of the ear nodules might be useful as a conveniently observable index of the degree of arterial involvement. This proved not to be the case. In some instances in which the ear nodules were large, arterial lipid deposits were scanty and vice versa. No very close correlation could be detected between the size of the nodules and the degree of hypercholesterolemia. If the ears of one rabbit were pierced by several tags at the same time, nodules developed at the edges of some but not others, and the size of the nodules varied greatly. It is evident, therefore, that the character and extent of local tissue injury at the site of the ear tag played an important role in the development of the lesions. It is possible that if the intensity of injury produced mechanically was sufficiently standardized, the degree of xanthomatous reaction provoked might be more closely related to arterial lipid deposition.

In a group of 6 rabbits, piercing earrings of a uniform size were

used. The catch on these could be screwed against the skin surface to about the same degree of tightness in every instance. The marginal nodules that developed, however, still varied unpredictably in size.

*Relation of Xanthogranulomatous Aural Nodules  
to Sebaceous Glands*

The excessive deposition of doubly refractile, extracellular free cholesterol in the ear nodules indicated that some special mechanism was the basis for its accumulation. There exists the possibility that the sebaceous glands in a given locality have a constant secretion rate of cholesterol-containing sebum or cerumen and that trauma by the metal tags permits the escape of lipid substance into the periglandular tissue to form an expanding deposit. In support of this view, it was noted that focal accumulations of lipid at the margins of the nodules occasionally appeared to be directly related to traumatized sebaceous glands. The sebum within the glands resembled the lipid masses in the nodules in both polarized light and in Sudan IV stained frozen sections.

Inflammatory lesions occurring in noncutaneous tissues in hypercholesterolemic rabbits occasionally contained abnormal amounts of lipid so that it was impossible to be certain that the lipid in the aural lesions was derived from sebaceous glands. However, with the possible exception of pneumonic lesions,<sup>5</sup> other visceral inflammatory foci in these animals usually contained less lipid and certainly very much less doubly refractile, non-sudanophilic substance than the aural lesions. Chronic pneumonic inflammations frequently contain large numbers of lipid-laden phagocytes even in animals with normal blood cholesterol levels. The excessive amounts sometimes found in these lesions in hypercholesterolemic rabbits may be an exaggeration of a fairly regular feature of such processes.

If it could be proved that the lipid masses in the aural lesions were derived in part from sebaceous glands, it would indicate that the secretory activity of these glands might be increased in cholesterol-fed rabbits. According to Rothman and Schaaf,<sup>6</sup> the amount of cholesterol found in cutaneous sites is increased when there is pathologic overloading of the system with this substance. It is surprising that so little attention has been paid to the role of the skin as an excretory route of cholesterol. The content of cholesterol and related substances such as squalene in sebum is variable in different species and skin regions.<sup>7</sup> The amount, however, is relatively large compared to other body fluids or secretions (3 to 4 per cent, Lorincz and Stoughton<sup>7</sup>; 15 per cent, Deuel<sup>8</sup>).

An attempt was made to produce xanthomatous nodules by inserting ear tags in the abdominal skin and that of the back. Unfortunately, most of the tags became detached or sloughed, leaving ulcerated lesions which healed without obvious nodule formation. No nodular swellings were found at the margins of metal tags which remained in position at these sites for several weeks. The cartilaginous plate in the rabbit's ear probably provides suitable anchorage so that when the metal tag is pressed against it, the intervening skin may be injured without undergoing necrosis. It is possible, however, that the secretory activity of the cerumen-producing, aural sebaceous glands is greater than that of glands in other skin areas.

#### *Regression of Xanthogranulomatous Nodules After Removal of Ear Tags*

If the ear tags were removed after nodular thickenings had formed, there was a gradual reduction in the size of the nodules and definite healing occurred even though cholesterol feeding was continued. The areas of scarring had a saucered appearance and were very hard. Sections through healed lesions revealed almost no doubly refractile or sudanophilic substance or, at most, only small localized collections, often at a distance from the center of scarring. Associated with fibrosis, zones of irregular cartilaginous proliferation were found about the margins of the perforated cartilaginous plate, and foci of bone formation were common.

In a group of 12 rabbits, cotter pins, 2 mm. in diameter, were inserted through the ear 4 weeks after cholesterol feeding was begun and were permitted to remain in position for 2 weeks. This was done in order to determine whether simple perforation of the skin and cartilaginous plate would cause nodules or whether pressure of a flat metal disc against the skin surface was an essential. The perforations healed within a week or two after the pins had been removed, leaving only minute, dimpled depressions in the skin surface. Frozen sections of such areas, however, occasionally demonstrated small masses of lipid at the margins of the scars.

#### *Resorption of Lipid from Aural Nodules After Discontinuation of Cholesterol Feeding*

After a 10-week period of cholesterol feeding, 8 rabbits with prominent ear nodules were permitted to eat normal stock diet for an additional 10 weeks, the ear tags remaining in place. Very little alteration occurred in the size of the nodules during this interval,

although the blood cholesterol fell markedly after 3 weeks on normal diet and attained normal level after 7 weeks.

When sacrificed, the 8 rabbits had varying degrees of aortic lipid deposition. The deposits were about as extensive as would have been found at the end of the cholesterol feeding period. In other words, there was no evidence that appreciable amounts of lipid had been resorbed from the vessels. Histologic examination of the aural nodules, however, indicated that their lipid content was very much less than that regularly observed in nodules removed at the end of cholesterol feeding. Apparently as the blood cholesterol levels fell, the lipid within the nodules diminished. This was probably effected by fibrous tissue replacement rather than resorption since the nodules did not shrink appreciably in size. The lipid deposits in large arteries showed only slight evidence of fibrous replacement during the same 10 weeks' interval on normal diet. Apparently, conditions necessary for the proliferation of fibrous tissue are greater in the skin than in the arterial intima. A comparable observation was made when the histologic structure of spontaneous human cutaneous xanthomas was compared to that of arterial atheromatous plaques.<sup>1</sup> It was felt that growth of capillaries into cutaneous lipid deposits was more easily achieved than into arterial atheromatous plaques, and as a consequence, fibroblastic activity in the former was greater.

#### SUMMARY

The accumulation and removal of lipid deposits in the skin of rabbits made hypercholesterolemic by cholesterol feeding were investigated under different circumstances. If hypercholesterolemic rabbit serum was introduced into intact skin with minimal trauma, it was rapidly resorbed, leaving no residual lipid deposit. This indicated that the interstitial fluid was not so saturated with cholesterol that it was incapable of transporting additional cholesterol introduced locally. If, however, continued trauma was applied and granulation tissue formation was established in the skin, as at the margins of metal ear tags, lipid in large amounts accumulated. The bulk of these lipid masses was probably free cholesterol since it was non-sudanophilic, crystalline, extracellular, doubly refractile and freely soluble in fat solvents. It is suspected that part of this lipid mass was derived from traumatized sebaceous glands.

If the metal ear tags were detached and the source of irritation thus removed, healing with resorption of the lipid deposit occurred even if the rabbit remained hypercholesterolemic. If the ear tag was



permitted to remain in place but cholesterol feeding was discontinued, the nodular swellings persisted but the lipid content diminished as the blood cholesterol lessened. These observations indicate that the ability to mobilize and remove cholesterol may vary at different tissue sites. Cholesterol is relatively easily removed from skin but only poorly from the arterial wall.

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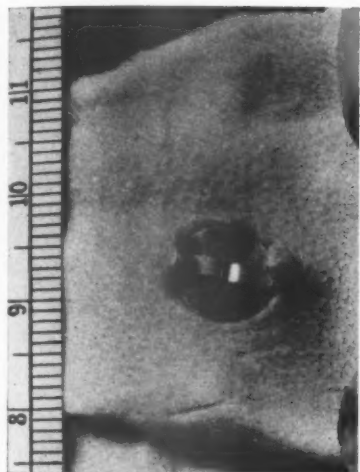
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[ Illustrations follow ]

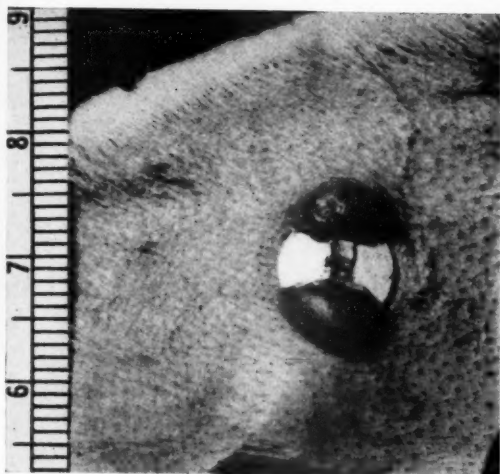


## LEGENDS FOR FIGURES

FIGS. 1 to 5. Photographs of nodular lesions at margins of ear tags. The small excrescences at the edges of the tag in Figure 1 illustrate the type of lesions frequently seen in rabbits on normal diets. Figure 2 shows enlargement of such a nodule as the result of lipid deposition after 6 weeks of cholesterol feeding. Figures 3 to 5 illustrate progressive enlargement of the nodular swellings in cholesterol-fed rabbits until the entire tag is enclosed in lipid-rich granulation tissue, as in Figure 5.



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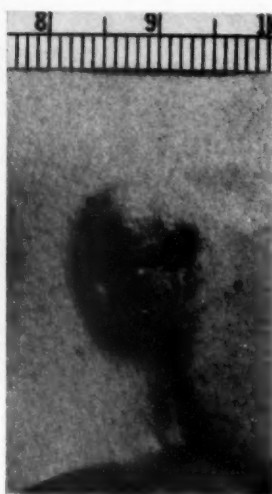
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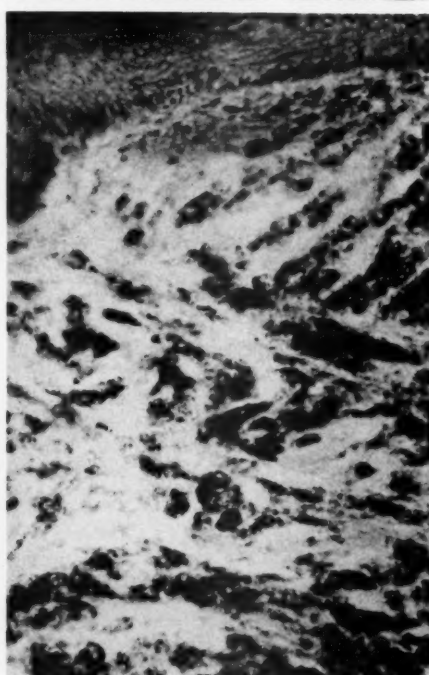
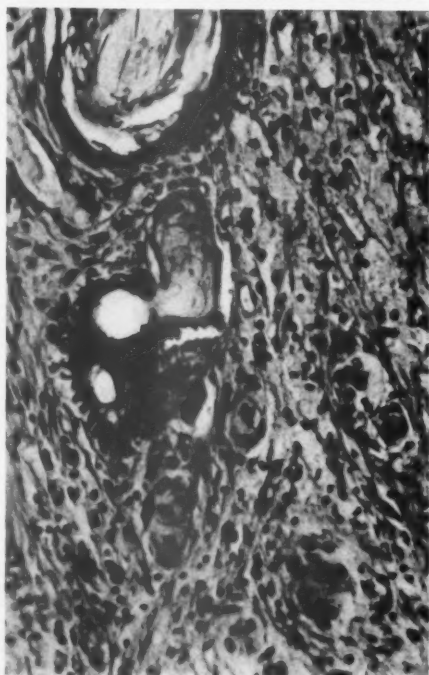
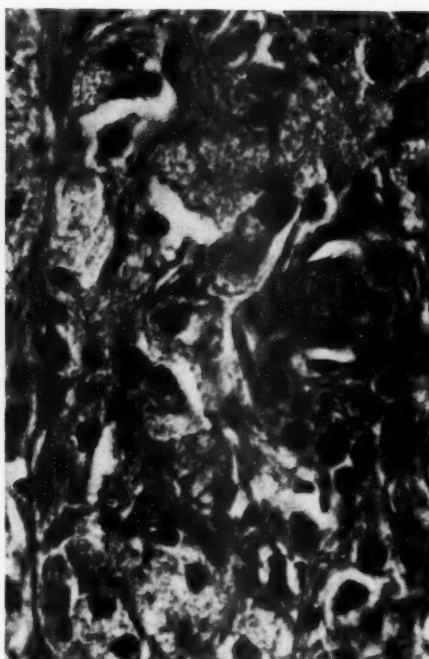
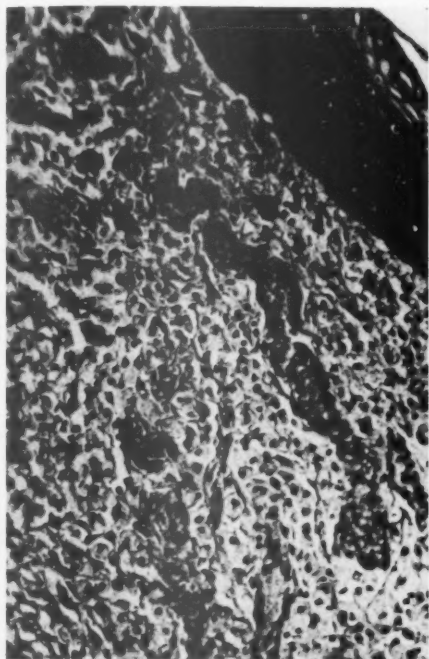


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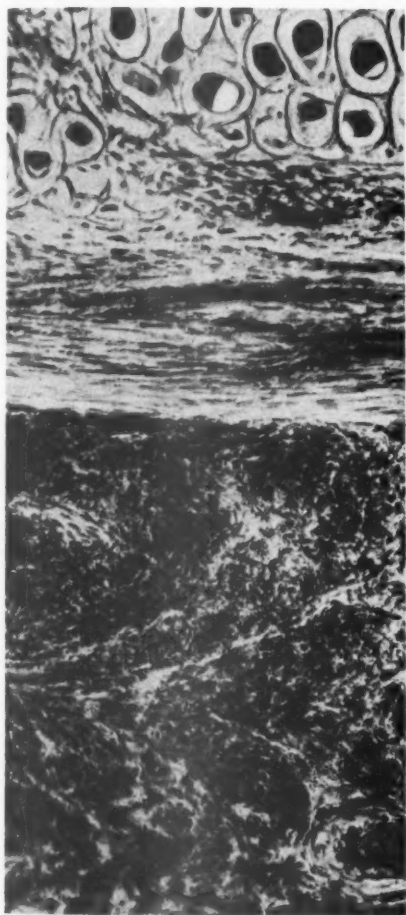


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- FIG. 6. Lipid containing phagocytes in ear nodule. Engorged capillaries are prominent throughout such lesions. Hematoxylin and eosin stain.  $\times 138$ .
- FIG. 7. High power photomicrograph of lipid-containing phagocytes, showing the finely loculated cytoplasm of these cells. Hematoxylin and eosin stain.  $\times 409$ .
- FIG. 8. Remnants of a damaged sebaceous gland and hair follicle are surrounded by masses of lipophages. Hematoxylin and eosin stain.  $\times 157$ .
- FIG. 9. Frozen section of ear nodule stained with Sudan IV. The black material is sudanophilic lipid contained largely within the lipophages.  $\times 125$ .



FIGS. 10 and 11. Duplicate photomicrographs. A Sudan IV stained frozen section of a large ear nodule. Figure 11 is taken by means of polarized light. In addition to sudanophilic material that appears blackish gray in Figure 10, there is a large amount of nonstainable doubly refractile, crystalline, extracellular lipid material in Figure 11. The large fat droplets in the cartilage cells in the aural cartilage plate at the upper end of the photograph are not doubly refractile.  $\times 117$ .



10



11

FIG. 12. Low power photomicrograph of an ear nodule, utilizing polarized light. The light areas represent an abundant deposit of doubly refractile cholesterol crystals. These have formed on both sides of the central auricular cartilaginous plate. Frozen section stained with Sudan IV.  $\times 11$ .

FIG. 13. Photomicrograph of an irregular nodule which formed in the ear of a cholesterol-fed rabbit at the margins of an ear tag after 7 weeks. The tag was then removed, and the nodule subsequently reduced in size and became irregular in outline although cholesterol feeding was continued for 5 additional weeks. Histologically, the lesion consists chiefly of dense fibrous tissue containing relatively little sudanophilic material. Frozen section stained with Sudan IV.  $\times 6$ .

FIG. 14. Duplicate photomicrograph of Figure 13, taken in polarized light. Relatively little doubly refractile material is present and this is located at the margins of the fibrous nodules.  $\times 5$ .

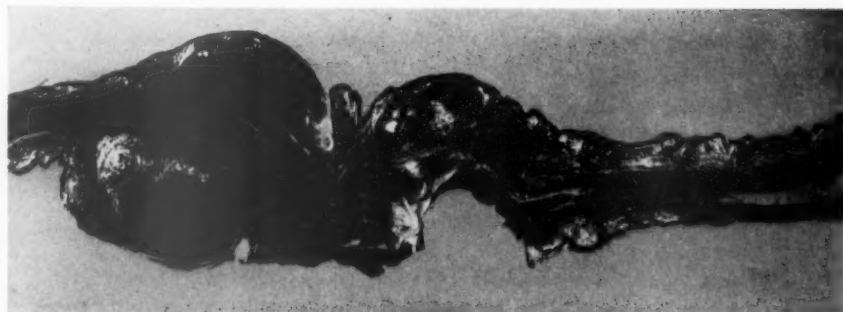




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## EFFECT OF TESTOSTERONE ON TESTICULAR LESIONS PRODUCED BY DL-ETHIONINE IN RATS\*

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The hepatic alterations induced in rats by DL-ethionine are to a certain extent dependent on the sex of the animal. Characterized by fatty infiltration of the liver, this experimental lesion occurs only in female rats or castrated males.<sup>1,2</sup> Moreover, implantation of testosterone pellets in castrated males prevents these fatty changes.<sup>2</sup> The presumed protein-sparing effect of testosterone may constitute the mechanism by which normal male rats escape the hepatic damage which follows the administration of ethionine.<sup>1</sup>

Ethionine also causes severe testicular injury in rats.<sup>3-5</sup> We have investigated these lesions and have reported them in an earlier paper.<sup>6</sup> The present study has as its purpose the determination of whether testosterone serves to protect against the induction of the testicular lesions as it does against lesions in the liver.

### MATERIAL AND METHODS

Twenty male albino rats of a random-bred strain (Hebrew University strain), weighing  $120 \pm 20$  gm. were used in this experiment. The animals were maintained on a diet of Purina Laboratory Chow (Ralston Purina Company, St. Louis, Missouri) and drinking water *ad libitum*. Each rat was kept in a separate metabolic cage.

The rats were divided into 4 groups of 5 animals each (Table I), as follows: Groups 1 and 2 were offered DL-ethionine (Nutritional Biochemicals Corporation, Cleveland, Ohio) as a 0.5 per cent solution of drinking water. It was estimated that each rat ingested about 70 to 100 mg. of ethionine daily. In addition, the rats in group 1 received intraperitoneal injections of testosterone propionate, 2.5 mg. dissolved in 0.5 cc. of olive oil. The first injection was administered on the fourth day of ethionine ingestion, and subsequent injections were given twice weekly to a total dosage of 15 mg. until 2 days before the end of the experiment. The rats in group 2 received intraperitoneal injections of 0.5 cc. of olive oil alone at the same intervals. Group 3 was used as a control; these rats received intraperitoneal

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TABLE I  
Effect of Ethionine and Treatment with Testosterone: Testicular Injury and Weight Changes After 12 Days

|  | Group 1<br>P.L.C., ethio.,<br>and testost. | Group 2<br>P.L.C., ethio.,<br>and olive oil | Group 3<br>P.L.C., water,<br>and testost. | Group 4<br>P.L.C., water,<br>and olive oil | Group 5<br>P.L.C. and<br>ethio. | Group 6<br>P.L.C. and<br>water |
|--|--|---|---|--|---------------------------------|--------------------------------|
| Number of animals                                    | 4*   | 5   | 5   | 5  | 5                               | 5                              |
| Content of tubules<br>(average of 20 cross sections) |  |   |   |  |                                 |                                |
| Golgi phase  |  |   |   |  |                                 |                                |
| Stage I  | 4  | 4   |   |  | 1                               |                                |
| II   |  | 4   |   |  |                                 |                                |
| III  |  | 4   |   |  |                                 |                                |
| Cap phase  |  |   |   |  |                                 |                                |
| Stage IV   |  | 9   | 2   | 2  |                                 | 2                              |
| V  |  | 9   | 3   | 3  |                                 | 5                              |
| VI   |  | 9   | 1   | 1  | 9                               | 2                              |
| VII  |  | 9   | 3   | 2  |                                 | 4                              |
| Acrosome phase                                       |  |   |   |  |                                 |                                |
| Stage VIII†  |  | 9   | 3   | 3  |                                 | 3                              |
| IX   |  | 6   | 3   | 2  |                                 | 1                              |
| X  |  | 6   |   |  |                                 |                                |
| XI   |  | 6   |   | 1  | 7                               | 1                              |
| XII  |  | 6   | 2   | 1  |                                 |                                |
| XIII   |  | 6   | 2   | 2  |                                 | 2                              |
| XIV  |  | 6   |   | 2  |                                 |                                |
| Undifferentiated stage                               | 4  | 1   |   |  | 3                               |                                |
| Average diameter, tubules ( $\mu$ )                  | 174  | 170   | 200                                       | 210  | 180                             | 200                            |
| Residual bodies                                      | 4/20                                       | 5/20  | 11/20                                     | 9/20                                       | 3/20                            | 10/20                          |
| Spermatozoa  | 5/20                                       | very few                                    | 11/20                                     | 18/20                                      | very few                        | 11/20                          |
| Average weight change (%)                            | -12  | -15.4                                       | +21                                       | +33  | -14.5                           | +23                            |

P.L.C.: Purina Laboratory Chow.

ethio.: Ethionine, 0.5%.

testost.: Testosterone, 2.5 mg. intraperitoneally.

olive oil: 0.5 cc. intraperitoneally.

\* One animal was found dead on the fifth day of experiment.

† Stage VIII designated as cap phase pattern because of similarity to cap phase spermatids.

injections of testosterone in oil, but ethionine was not administered. Group 4 rats received injections of olive oil alone, and as in group 3, ethionine was not given.

On the twelfth day of the experiment, unilateral orchidectomy was performed, and the remaining testis was removed at the termination of the experiment on the 24th day. The testes procured from the animals in the 4 groups were compared with each other, with normal rat testes, and with testes containing lesions induced by ethionine in an earlier investigation.<sup>6</sup> The specimens were prepared for histologic examination as previously described.<sup>6</sup>

### RESULTS

#### *After 12 Days*

All the animals which received ethionine, including those treated with testosterone, lost weight during the first 12 days of the experiment. All those not receiving ethionine, gained from 21 to 33 per cent in weight (Table I). The testes of the rats in the ethionine-treated groups were smaller than the controls, and the diameters of the seminiferous tubules were reduced (170 to 180  $\mu$ ) as compared with the normal (200 to 210  $\mu$ ).

In the ethionine-treated animals receiving olive oil alone and no testosterone, the seminiferous tubules exhibited the same germ cell association patterns described in our previous study<sup>6</sup>; namely, (a) a Golgi phase pattern, affecting all germ cells up to Golgi phase spermatids; (b) a cap phase pattern, affecting germ cells up to the cap phase spermatids (giant cells were also found in these lesions); and (c) a pattern characterized by several layers of spermatocytes of different generations but an absence of all spermatids. Normally, acrosome phase spermatids should have been present in these germ cell associations. Instead, maturation failed to proceed beyond the cap phase spermatid stage, and free spermatozoa were not manifest.

In the group receiving testosterone, free spermatozoa were found in 25 per cent of the tubules examined. However, no development beyond the cap phase spermatids was observed, and the tubules were also reduced in diameter, in a manner similar to that observed in rats which did not receive testosterone. The Leydig cells and the intertubular tissue exhibited no significant alterations in response to ethionine treatment.

The control groups showed normal spermatogenesis and maturation, and the Leydig cells were without pathologic features.

TABLE II  
Effect of Ethionine and Treatment with Testosterone: Testicular Injury and Weight Changes After 24 Days

|  | Group 1<br>P.L.C., ethio.,<br>and testost. | Group 2<br>P.L.C., ethio.,<br>and olive oil | Group 3<br>P.L.C., water,<br>and testost. | Group 4<br>P.L.C., water,<br>and olive oil | Group 5<br>P.L.C. and<br>ethio. | Group 6<br>P.L.C. and<br>water |
|--|--|---|---|--|---------------------------------|--------------------------------|
| Number of animals                                    | 4  | 5   | 5   | 5  | 4*                              | 5                              |
| Content of tubules<br>(average of 20 cross sections) |  |   |   |  |                                 |                                |
| Golgi phase  |  |   |   |  |                                 |                                |
| Stage I  |  |   |   |  |                                 |                                |
| II   |  |   | 1   |  |                                 | 1                              |
| III  |  |   |   |  |                                 |                                |
| Cap phase  |  |   |   |  |                                 |                                |
| Stage IV   |  |   | 3   | 5  |                                 | 1                              |
| V  |  |   | 2   | 5  |                                 | 5                              |
| VI   |  |   | 1   |  |                                 | 1                              |
| VII  |  |   | 3   | 3  |                                 | 2                              |
| Acrosome phase                                       |  |   |   |  |                                 |                                |
| Stage VIII†  |  |   | 2   | 2  |                                 | 4                              |
| IX   |  |   | 2   | 1  |                                 | 1                              |
| X  |  |   | 1   |  |                                 | 1                              |
| XI   |  |   | 1   |  |                                 | 1                              |
| XII  |  |   | 1   |  |                                 | 1                              |
| XIII   |  |   | 2   |  |                                 | 1                              |
| XIV  |  |   | 1   | 3  |                                 | 3                              |
| Undifferentiated stage                               |  |   |   |  |                                 |                                |
| Average diameter, tubules ( $\mu$ )                  | 126  | 175   | 252                                       | 252  | 96                              | 280                            |
| Residual bodies                                      |  |   | 9/20                                      | 9/20                                       |                                 | 8/20                           |
| Spermatozoa  |  |   | 14/20                                     | 14/20                                      |                                 | 15/20                          |
| Average weight change (%)                            | -12.7                                      | -22   | +37                                       | +52  | -23                             | +37                            |

P.L.C.: Purina Laboratory Chow.

ethio.: Ethionine, 0.5%.

testost.: Testosterone, 2.5 mg. intraperitoneally.

olive oil: 0.5 cc. intraperitoneally.

\* Anesthetic death of one animal during left orchidectomy on the twelfth day of experiment.

† Stage VIII designated as cap phase pattern because of similarity to cap phase spermatids.

*After 24 Days*

All ethionine-treated rats lost weight ranging to 23 per cent of the original weight; the controls gained as much as 50 per cent of the initial weight (Table II). Their testes were even smaller in proportion to the controls than at the 12 day period. The diameters of the seminiferous tubules varied widely from 96 to 175  $\mu$ ; those of the controls were 250 to 280  $\mu$  and varied only slightly (Table II).

Administration of testosterone did not prevent the severe tubular lesions induced by ethionine. In typical cross sections of tubules there were practically no germ cells, spermatozoa or residual bodies. The basement membrane was lined almost exclusively by Sertoli cells, and germinal epithelium was replaced by pale, hematoxylin-staining, undifferentiated fibrils.

The Leydig cells in rats receiving both testosterone and ethionine showed severe atrophy (Fig. 1). This was in marked contrast to the Leydig cell hyperplasia observed in animals receiving ethionine alone<sup>6</sup> (Fig. 2).

All control rats exhibited normal spermatogenesis (Table II), and Leydig cells were normal in size and number. It was noted that the rats receiving ethionine secreted much less urine than the normal controls.

## DISCUSSION

In our experiments no lesions appeared in the testes of rats receiving testosterone alone. Most authors, however, have indicated that testosterone causes Leydig cell atrophy and some damage to the tubular epithelium.<sup>7-9</sup> The discrepancy in observations may be attributable to the fact that we administered testosterone only twice weekly. Thus there were intervals during which most of the testosterone could have been eliminated from the body, permitting intermittent recovery of the testis from whatever damaging effect the hormone may have had.

At 12 days the animals receiving ethionine exhibited arrest of maturation of germ cells at the level of cap phase spermatids whether or not testosterone was administered. In those instances in which both ethionine and testosterone were administered, however, mature spermatozoa were encountered in some of the seminiferous tubules. This was not the case if ethionine alone was administered. It is possible that testosterone exerts a protective effect on mature spermatozoa. Since no stages intermediate between cap phase spermatids and mature spermatozoa could be demonstrated, one cannot determine whether testosterone had a protective effect early in the course of the experiment. When ethionine administration was continued for



24 days, however, testosterone appeared to have no effect at all in modifying its injurious action.

The severe atrophy of the Leydig cells which was noted after 24 days of exposure to both ethionine and testosterone appears significant in view of the fact that these elements became hyperplastic when ethionine alone was administered.<sup>6</sup> It is possible that the reduction in urinary excretion in rats receiving ethionine might have contributed to a retention and accumulation of testosterone and its metabolites, thus permitting them to reach injurious levels.<sup>7,10</sup>

Our experiments indicate, therefore, that the administration of testosterone affords some protection against the injurious effects of ethionine, but does not protect the testis to the extent that it does the liver.

#### SUMMARY

The effect of testosterone on testicular lesions caused by the administration of ethionine to rats was investigated. After 12 days of exposure to ethionine, rats exhibited no maturation of germinal epithelium beyond cap phase spermatids. Rats simultaneously receiving testosterone, however, showed mature spermatozoa in a few seminiferous tubules.

After 24 days of exposure to both ethionine and testosterone, the latter proved to be ineffective in preventing complete destruction of the seminiferous epithelium. Indeed, animals receiving testosterone showed severe atrophy of the Leydig cells, while those receiving ethionine alone exhibited hyperplasia of these elements. It is apparent that testosterone affords no protection against the injurious effects of ethionine in the rat testis.

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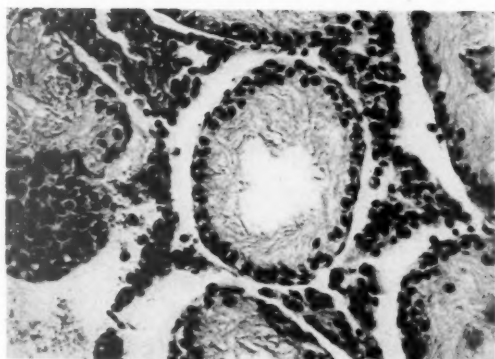
The authors are indebted to Dr. L. Schindel, "Teva" Middle East Pharmaceutical & Chemical Works Ltd., Jerusalem, Israel, who provided the testosterone propionate. The technical assistance of Mrs. Cilla Perper and Mrs. Ruth Ungar is acknowledged. The photomicrographs were made by Mrs. Hanah Weinman and Mr. Haim Reubeni.

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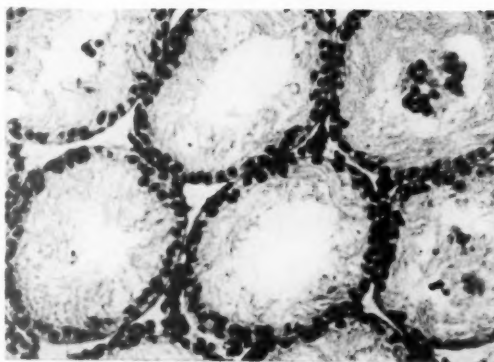
[ Illustrations follow ]

## LEGENDS FOR FIGURES

- FIG. 1. Seminiferous tubules from a rat ingesting ethionine for 24 days. Note complete destruction of germinative epithelium and marked hyperplasia of Leydig cells. Hematoxylin and eosin stain.  $\times 200$ .
- FIG. 2. Seminiferous tubule from a rat ingesting ethionine for 24 days and receiving injections twice weekly of 2.5 mg. of testosterone propionate. Note severe atrophy of Leydig cells. Hematoxylin and eosin stain.  $\times 200$ .



1



2



FIFTY-SIXTH ANNUAL MEETING  
OF THE  
AMERICAN ASSOCIATION OF PATHOLOGISTS  
AND BACTERIOLOGISTS

BOSTON, MASSACHUSETTS

APRIL 23RD, 24TH, AND 25TH, 1959





THE AMERICAN ASSOCIATION OF PATHOLOGISTS  
AND BACTERIOLOGISTS

Fifty-sixth Annual Meeting

HOTEL SOMERSET

Boston, Massachusetts

April 23rd, 24th, and 25th, 1959

PRESIDENT MORITZ IN THE CHAIR

BUSINESS MEETING

The following nominations for elective officers were submitted by the Council:

|                                   |                             |
|-----------------------------------|-----------------------------|
| <i>President</i>                  | DR. DOUGLAS H. SPRUNT       |
| <i>Vice-President</i>             | DR. JOHN D. KIDD            |
| <i>Secretary</i>                  | DR. RUSSELL L. HOLMAN       |
| <i>Treasurer</i>                  | MAJOR GEN. ELBERT DECOURSEY |
| <i>Incoming Member of Council</i> | DR. JEROME T. SYVERTON      |

Additional nominations were called for. None having been offered, it was moved and seconded from the floor that the Secretary be instructed to cast a unanimous ballot for the entire slate.

The President commented on the purposes of the Association as outlined in the Constitution and explained briefly the requirements for membership based on long-established custom. He then directed the Secretary to report the following actions of the Council:

Election of New Members

|                        |                          |
|------------------------|--------------------------|
| Renato L. Baserga      | Robert Dana Langdell     |
| Ivan L. Bennet, Jr.    | Francisco V. Lichtenberg |
| Walter Russell Benson  | Raul A. Marcial-Rojas    |
| J. Owen Blache         | D. D. Mark               |
| L. W. Diggs            | Donald G. McKay          |
| George Edward Foley    | Hans Meier               |
| W. S. Gilmer           | Abe Oyamada              |
| Fairfield Goodale, Jr. | Gordon Barry Pierce      |
| Edith Grishman         | Giuseppe Della Porta     |
| Ferenc Gyorky          | Philip H. Prose          |
| Donald West King, Jr.  | Herschel Sidransky       |
| Janis V. Klavins       | Cesar Someza             |

David Spiro  
Harlan J. Spjut  
Richard L. Swarm

Charles Bruce Taylor  
Gordon F. Vawter  
Daniel Leigh Weiss

Re-election of Members of Editorial Board  
and Assistants to Officers

*Assistant Secretary* . . . . . Dr. Jack P. Strong  
*Assistant Treasurer* . . . . . Dr. Elson B. Helwig  
*Editorial Assistant* . . . . . Miss Janet E. Smith

*Member of the Editorial Board*

Dr. R. Philip Custer . . . Term to expire December 1965

With deep regret, the recording of the deaths of:

|                     |                       |
|---------------------|-----------------------|
| Joseph D. Aronson   | Elise S. L'Esperance  |
| Frederick I. Dessau | James S. McCartney    |
| N. Chandler Foot    | Joseph W. McMeans     |
| Edwin S. Gault      | Donald J. Rehbock     |
| Adolph Hochwald     | Siegfried Tannhauser  |
| Ernest E. Irons     | Friedrich J. Wohlwill |

The question of resignations and retirements postponed from last year was cleared by the following legislative action. The following amendment to the bylaws was read at the Business Meeting last year:

Bylaw 6 (dealing with method of amendment) was to become Bylaw 7.

Bylaw 6 (new)

(a) Members in good standing who have attained age 65 or who have retired from gainful professional activity because of physical disability may, upon application to Council, be granted emeritus membership.

(b) Emeritus members shall remain upon the rolls of the Association and shall receive regular notices. They shall, however, be relieved of payment of dues and may neither hold office nor receive *The American Journal of Pathology* except by independent subscription.

The Secretary moved the adoption of the new bylaws. This was seconded from the floor and passed unanimously.

The Secretary announced that the next Annual Meeting will be held in Memphis, Tennessee, April 28, 29, and 30, 1960. The topic for the symposium will be "Genetic Factors in Disease." The referee will be Dr. Nash Herndon.

The Secretary further announced that the place of the Annual Meeting for 1961 had not been definitely determined. The topic for the Symposium will be "Disorders of Fine Structure."

Dr. Douglas H. Sprunt, President-elect and Chairman of the Program Committee for 1960, was requested to present the decisions of Council regarding the program for next year.

1. The Program Committee will consist of the President (Chairman), Vice-President, Secretary, and Editor, as it has in the past.
2. Announcement of the meeting will be sent out early (around November 15, 1959) and the final date for the receipt of abstracts will be approximately January 15, 1960.
3. Number of papers selected for oral presentation will be about the same as this year, i.e., ample for five single sessions (including the Symposium).
4. Time for presentation of papers will not exceed 10 minutes, except at the discretion of the Program Committee. Discussion will be encouraged.
5. Abstracts, including those "Read by Title," will be published in the Program to be mailed out to the members approximately one month before the Annual Meeting. Abstracts will not be published in *The American Journal of Pathology*.

Dr. Edward A. Gall, the Editor, announced that efforts were under way to change *The American Journal of Pathology* from a bi-monthly to a monthly publication, effective January, 1960. This change, coupled with the omission of abstracts from The Journal, will permit an increase in the number of articles published and also will shorten the period between receipt and publication of accepted manuscripts.

The Secretary reported that a revised Roster of Membership was in progress and would be mailed to the members some time this year.

The President then asked if there was any new business from the floor. An objection to the revised program introduced this year, namely, single instead of double sessions, was voiced from the floor. President Moritz gave assurance that the objection would receive appropriate consideration by the Council.

The Business Meeting adjourned at 2:35 P.M.

Russell L. Holman, *Secretary*

## REPORT OF THE TREASURER

The report of the Treasurer was submitted to the Council and accepted. It was accompanied by a letter of certification from Ralph Cole, Certified Public Accountant, of Washington, D.C. In condensed form, the Treasurer's report follows:

## General Checking Account

*Receipts*

|   |             |
|---|-------------|
| Balance on hand, January 1, 1958.....   | \$ 6,744.32 |
| Transfer of funds, Riggs National Bank Savings Account.....   | \$ 1,261.00 |
| Certificate of savings and loan association cashed.....   | 5,000.00    |
| Membership dues.....  | 13,730.00   |
| International Academy of Pathology.....   | 88.48       |
| Interest on bonds and savings accounts and dividends on shares with building and loan associations..... | 1,295.16    |
|   | <hr/>       |
|   | 21,374.64   |
| Total receipts.....   | <hr/>       |
|   | \$28,118.96 |

*Disbursements*

|  |             |
|--|-------------|
| American Journal of Pathology.....                   | \$10,984.00 |
| Secretary's office, clerical.....                    | \$ 200.00   |
| Printing, supplies, miscellaneous.....               | 1,340.48    |
|  | <hr/>       |
|  | 1,540.48    |
| Treasurer's office, bonding and auditing.....        | \$ 165.00   |
| Secretarial services.....                            | 225.00      |
| Printing, supplies, miscellaneous.....               | 321.58      |
|  | <hr/>       |
|  | 711.58      |
| Micellaneous, annual meeting.....                    | \$ 79.78    |
| Intersociety Committee on Pathology Information..... | 500.00      |
| National Society for Medical Research.....           | 50.00       |
| Purchases of stocks.....                             | 11,851.63   |
|  | <hr/>       |
|  | 12,481.41   |
| Total disbursements.....                             | <hr/>       |
|  | \$25,717.47 |
| Balance on hand, December 31, 1958.....              | <hr/>       |
|  | \$ 2,401.49 |

## Investment Inventory

## Savings Accounts

|  |             |
|--|-------------|
| First and Citizens National Bank, Alexandria, Va.....  | \$ 5,000.00 |
| Riggs National Bank, Washington, D.C.....              | 4,993.47    |
| National Bank of Washington, D.C.....                  | 3,591.87    |
| Home Savings & Loan Association, Los Angeles.....      | 5,000.00    |
| Olympic Savings & Loan Association, Berwyn, Ill.....   | 5,000.00    |
| Mutual Savings & Loan Association, Pasadena, Calif.... | 5,000.00    |

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 \$28,585.34

## Stocks

|  |             |
|--|-------------|
| 200 Shares, Tri-Continental Corporation..... | \$ 6,261.00 |
| 200 Shares, Adams Express Company.....       | 5,590.63    |

---

 11,851.63

|  |             |
|--|-------------|
| Total of investment inventory.....                 | \$40,436.97 |
| General checking account, Riggs National Bank..... | 2,401.49    |

|                    |             |
|--------------------|-------------|
| Total assets ..... | \$42,838.46 |
|--------------------|-------------|

Elbert DeCoursey, *Treasurer*



## SCIENTIFIC PROCEEDINGS

### ABSTRACTS

THE HISTOLOGIC EXAMINATION OF SURGICALLY RESECTED PULMONARY GRANULOMAS, UTILIZING GOMORI'S METHENAMINE-SILVER NITRATE STAIN. Edward L. Segal, Grier F. Starr, and Lyle A. Weed,\* Mayo Clinic and Mayo Foundation, Rochester, Minn.

Since September, 1946, all surgically resected pulmonary granulomas at the Mayo Clinic have been subjected to a thorough microbiologic and histologic examination. In spite of this extensive investigation, the cause of approximately 60 per cent of the pulmonary granulomas could not be determined. For the past 2½ years, Gomori's methenamine-silver nitrate stain (GMS) for fungi has been employed as part of the routine examination of granulomas. In approximately 50 per cent of surgically resected pulmonary granulomas, structures resembling *Histoplasma capsulatum* were observed in GMS-stained sections of the granulomas. In only one case did culture produce *Histoplasma capsulatum*; cultures of the other granulomas in this group were negative. In approximately 10 per cent of the surgically resected pulmonary granulomas, structures resembling *Coccidioides immitis* were observed in GMS-stained sections; in half of these cases, *Coccidioides immitis* was cultured. These culturally negative structures resembling organisms were thought to be degenerated nonviable fungi which had lost their stainability with respect to hematoxylin and eosin. With the assumption that the structures observed with the GMS stain were fungi, the indeterminate group of surgically resected pulmonary granulomas was reduced to 17 per cent.

Additional studies revealed that the GMS stain was vastly superior to the periodic acid-Schiff stain for recognizing the structures believed to be fungi in tissue. These structures could be found more rapidly and under lower magnification with the GMS stain than with other staining methods. In many granulomas, structures resembling fungi were demonstrated with the GMS stain while they could not be observed with other staining techniques.

MULTIPLE MINUTE PULMONARY TUMORS RESEMBLING CHEMODECTOMAS. D. Korn, K. Bensch, A. Liebow,\* and B. Castleman,\* Massachusetts General Hospital, Harvard Medical School, Boston, Mass., and Yale University School of Medicine, New Haven, Conn.

It is the purpose of this paper to describe as an entity a distinctive, minute, pulmonary tumor which has not been recognized previously. The tumors tended to be multiple and to vary from microscopic size to a diameter of several millimeters. They were found in all lobes of the lung, and appeared not to be associated with any particular pulmonary or systemic pathologic process. Grossly, they were minute, sharply circumscribed, gray-white nodules and were most prevalent at the periphery of the lung. Histologically, the lesions developed strictly within the interstitial tissues of the lung in intimate relation to pulmonary veins. They had an organoid structure and exhibited a pattern of rounded nests of plump oval cells bounded by capillaries and delicate agyrophilic fibers.

From reconstruction studies based on serial sections, the capillaries were shown to be derived, at least in part, from pulmonary arteries. A blood supply from systemic arteries was not established, but may exist. Drainage proceeded into the pulmonary veins, athwart which these tumors lay. Precapillary anastomoses

\*Asterisks indicate members of The American Association of Pathologists and Bacteriologists. All others appear on the program "by invitation."



between pulmonary arteries and veins were found in association with the lesions.

There were a number of characteristic features which served to distinguish these tumors from previously known benign pulmonary neoplasms and atypical proliferative lesions. Although their precise nature remains undetermined, morphologic evidence suggests a resemblance to chemodectoma. The implication that the tumors may arise from normal intrapulmonary chemoreceptor organs is not inconsistent with the physiologic data supporting the existence of such organs in the lungs, even though they have never been identified anatomically. It is our impression that the proliferative lesions presented are not uncommon.

**GLOMOID HYPERPLASIA OF THE PULMONARY VASCULATURE; A PHENOMENON IN SEVERE PULMONARY HYPERTENSION.** Lawrence J. McCormack,\* Cleveland Clinic, Cleveland, Ohio.

Glomoid hyperplasia may be defined as the appearance of tangled, hypercellular masses of small blood vessels within the lumens of small arteries, occasionally veins, and occasionally appearing free within alveolar walls. Little attention has been paid to this structural alteration, which is associated with extreme degrees of hypertension of the lesser circulation. The accumulated literature is controversial on all aspects of the problem, but especially in respect to the age grouping of the patients and the intrapulmonary location of the lesion.

It is our purpose to discuss the nature of this vascular abnormality in the 16 instances in which the lesion occurred among 38 patients with congenital heart disease and pulmonary hypertension. This group was selected from 105 necropsies of patients with congenital heart disease. The exact nature of the lesion was not apparent until studies of serial sections were performed on lung specimens of the 5 cases with the greatest severity. It then became apparent that two types of lesions with similar appearance were manifest on random section. The first occurred entirely within arterial lumens, apparently representing a segmental proliferation of the vascular intima. The second, a much more complex lesion, had an intricate structure and was characterized by appearance free in the pulmonary parenchyma in random sections. This lesion possessed an arterial supply that terminated abruptly in a glomoid, tangled mass of vessels of capillary size, which in turn terminated equally abruptly in a pulmonary vein. As a result of the bulging of this mass into the lumen of the vein, the lesion appeared to originate within that vessel. It is possible that the abnormalities of this second type represent some form of a hyperplastic arteriovenous shunt.

A summary of the correlations with the types of congenital heart disease and pulmonary arterial pressures and a discussion of the possible fate of some of the lesions form parts of the presentation.

**PATHOLOGY OF PRIMARY PULMONARY HYPERTENSION. A STUDY BY SERIAL SECTIONS.** Javier Arias-Stella,\* Dante Penaloza and José Severino, Facultad de Medicina, Lima, Peru.

Representative blocks from each pulmonary lobe were studied by serial sections in 5 patients with primary pulmonary hypertension. All 5 were women ranging from 17 to 40 years of age. In none was there evidence of embolism or significant organic disease elsewhere. The severer lesions were located in the small muscular pulmonary arteries and arterioles. Intimal thickening, medial hypertrophy of variable degree, and so-called "plexiform," "angiomatoid," or "glomus-like" structures were widespread and constant lesions. Necrotizing arteritis was found in 2 cases as an isolated alteration.

Thin-walled arterioles, arising at right angles from medium-sized or small pulmonary arteries were very conspicuous at the point where the latter normally attained arteriolar caliber. Their proper evaluation was fundamental in the understanding of the histogenesis of the lesions.

Serial sections showed that intimal thickening originated at the point of

arterial branching, particularly at the site of origin of the thin-walled vessels. As an early alteration, marked endothelial proliferation, which formed polypoid masses, occurred at the origin of the thin-walled vessels. From these, new vascular channels were rapidly formed. Proliferated cells and new capillaries grew, filling the lumens of the arterioles constituting the so-called "plexiform lesions." Occasionally, newly formed capillaries separated the muscle in the arterial wall or distended the point of origin of the thin-walled vessels, giving rise to structures which have been erroneously interpreted as cicatrization following local necrosis. Later fibrous replacement of the richly vascularized tissue occurred in progressive fashion.

Sections in the regions from which the thin-walled vessels arose, but in which these branches were not evident, had an appearance identical with that described as arterial "medial hypoplasia" or "medial aplasia."

**CENTRILOBULAR EMPHYSEMA.** Fred Ionata and John P. Wyatt,\* St. Louis University School of Medicine, St. Louis, Mo.

After preparatory injection of colored latex into the vascular channels and intrabronchial insufflation with formalin, whole lung macrosections cut at 250  $\mu$  were prepared in the Gough manner. Of the 150 emphysematous lungs prepared, transilluminated, and scanned by this technique, it was found that approximately one half of these lungs showed a distinctive anatomic type of emphysema. Recognition of this characteristic morphologic pattern by conventional and current methods of post-mortem lung examination has been previously overlooked.

This type of emphysema may be localized or diffuse in extent, but the outstanding feature was the centrilobular anatomic site. The spatial orientation of the anatomic site for the basic lesion in centrilobular emphysema rests upon the recognition of the secondary lobule of Miller. Further exploitation of the centrilobular emphysematous lesions was readily accomplished by stereoscopy and serial histologic studies. These techniques allowed a detailed investigation of the basic structural alterations which were confined to respiratory bronchioles of the first, second, and third orders and were morphogenetically responsible for this pattern of emphysema. The precise and distinctive structural derangements of this type of emphysema were in sharp contrast to those observed in the terminal air sacs, recorded as the chronic vesicular or "panlobular" form of emphysema.

Because of anatomic similarity between diffuse centrilobular emphysema and the generalized focal emphysema observed in Southern Illinois soft coal miners, an inquiry into the relationship of environmental dust as a factor in the pathogenesis of non-occupational centrilobular emphysema is discussed. Clinical studies concerning functional appraisal of the cardiorespiratory disability and associations with chronic bronchitis are related.

**PRODUCTION OF BRONCHIAL CARCINOMAS IN MICE.** Shields Warren\* and Olive Gates,\* New England Deaconess Hospital, Boston, Mass.

Bronchial carcinomas are rare in mice, although alveolar adenomas occur often in some strains. Radiation is an effective carcinogenic agent in mice. Co<sup>60</sup> wire or Sr<sup>90</sup> glass beads were implanted by trocar into the lungs or pleural cavities of anesthetized mice. The animals tolerated these implants well. Sometimes, through misplacement or the production of local necrosis, the radioactive implants were found in adjacent muscle or bone. In the course of a year, tissues within a few millimeters of the implants received up to 100,000 r. Some mice died of pulmonary infection, others of paralysis secondary to radiation injury of the spinal cord, others of radiation reaction in the esophagus, and others of blood dyscrasias. We produced epidermoid carcinoma of the bronchus, as well as cancers of other tissues.

The bronchial and alveolar cells were quite resistant to the induction of

tumor by beta or gamma radiation, a tumor of more distant tissues, such as skin, being more readily induced.

**AGE CHANGES IN THE RENAL BASEMENT MEMBRANES OF RATS.** Charles T. Ashworth\* and Ralph R. Erdmann, The University of Texas Southwestern Medical School, Dallas, Texas.

Because of their potential physiologic and pathologic significance, the glomerular and tubular basement membranes were studied comparatively in infant (2 to 7 days old), young (4 to 6 months), and old (1.5 years) rats. Histologic, histochemical and electron microscopic studies were carried out. The basement membranes of infant rats mainly contained Alcian blue stainable material; PAS-positive substance was very scanty. With increasing age, a decrease in Alcian blue-positive and an increase in PAS-positive material was demonstrable. Electron microscopy showed that thickening of the membrane occurred progressively with increasing age. In infant rats the basement membrane contained a thin, dense, central lamina, and prominent lucent zones on each side, adjacent to the epithelial pedicles and to the endothelial cytoplasm. Comparable to the increased PAS-positive content of the basement membrane with age, the lucent zones decreased in prominence, almost disappearing completely in old rats, while the dense lamina increased in thickness.

**ACUTE ALTERATIONS PRODUCED BY URANYL NITRATE IN GLOMERULI OF RAT KIDNEYS; LIGHT AND ELECTRON MICROSCOPIC STUDIES.** Sergio A. Bencosme,\* Robert S. Stone, Harrison Latta\* and Sidney C. Madden,\* School of Medicine, University of California, Los Angeles, Calif.

As part of a systematic investigation of the ultrastructure of the kidney during physiologic and pathologic reactions, glomerular alterations in rat kidneys were studied during acute uranium poisoning.

With light microscopy, many large vacuoles and hyaline droplets were found in the glomerular epithelial cells. These were conceivably related to the polyuria and proteinuria which occurred in the animals. A striking lesion composed of tangled, rough fibers with collagen-like staining developed in the centrilobular region of most glomeruli after the third day. Collagen is not found in normal glomeruli.

With electron microscopy, the epithelium exhibited cytoplasmic vacuoles, hyaline droplets, other cytoplasmic bodies with varying types of internal membranes and granules, and myelin figures. There was also loss of foot processes, and replacing them were dense deposits in large portions of cytoplasm. No alterations were found in basement membranes or endothelial cells except possibly where they lay adjacent to the nodular centrilobular lesion. The latter contained typical collagen fibers, granular components, and an amorphous substance lying between the central dense layer of the basement membrane and the endothelium. In the midst of the lesion were cytoplasmic processes of intercapillary cells. The nature of these is controversial. The association of collagen with intercapillary cell processes is additional evidence that they may differ from endothelial cells.

**THE FINE STRUCTURE OF THE GLOMERULUS IN MEMBRANOUS GLOMERULONEPHRITIS (LIPOID NEPHROSIS).** Henry Z. Movat\* and Douglas D. MacGregor, University of Toronto, Toronto, Canada.

Until recently, membranous glomerulonephritis has been thought to represent a thickening of the basement membrane. Recent reports on electron microscopic studies state that the principal alteration consists of a loss of differentiation of the glomerular epithelial cells into foot processes. The findings in this investigation differ from both these views.

Tissue obtained by needle biopsy was examined by light and electron micro-

scopy. In the former, a delicate, irregular, acidophilic band of protein material was found to be deposited outside the basement membrane, and silver-positive clubs were noted in sections stained with silver methenamine. The electron micrographs showed a deposit between the basement membrane and the epithelial cells, the latter being pushed away, thus creating an appearance of "loss of foot processes." The deposit, corresponding to the one seen with the light microscope, varied in density. In a more advanced case, the basement membrane showed lighter and denser zones, while the glomerular epithelium exhibited vacuolization and the formation of hyaline droplets. All these changes were best seen in tissue fixed in osmium tetroxide and floated on a colloidal silver solution.

The alterations indicate that increased permeability of the basement membrane followed by exudation and precipitation of plasma protein between the basement membrane and the epithelium is the mechanism by which glomerular lesions develop in membranous glomerulonephritis. This view is in accord with observations of others with the fluorescent microscope, where a deposit of gamma globulin has been demonstrated along the basement membrane.

**TRIDIONE NEPHROSIS IN RATS.** D. B. Hackel,\* W. Heymann and J. L. P. Hunter, Western Reserve University, Cleveland Metropolitan General Hospital and Babies and Children's Hospital, Cleveland, Ohio.

The nephrotic syndrome has been reported as occurring in children during the course of prolonged Tridione therapy. The present experiments indicate that a similar disease can be produced in rats. Tridione solution was given orally to 21 rats 6 days a week, 38 to 57 mg. per dose, for as long as 18 months. Proteinuria was observed in 10 of the rats, beginning 2.5 to 14 months after the onset of treatment; hyperlipemia was found in all animals with proteinuria; hypoproteinuria occurred in 5; and azotemia developed in 3. Lesions were seen in the kidneys of all 21 rats, whether or not proteinuria occurred. The glomeruli showed irregular thickening of the basement membrane regions; this was best demonstrated in periodic acid-Schiff stained sections, and did not give an amyloid-like reaction with crystal violet. There was a moderate amount of sudanophilic material in the convoluted tubules, which contained eosinophilic, PAS-positive casts. Cortisone treatment had no detectable effect on the disease in 2 animals. Complement activity was normal in 13 rats, and agglutinating antibodies could not be detected in the serums of 10 animals.

**CALCIUM OXALATE RENAL CALCULI INDUCED IN RATS BY VITAMIN B<sub>6</sub> DEFICIENCY.** Stephen B. Andrus, Stanley N. Gershoff and Farouk F. Faragalla, Harvard School of Public Health, Boston, Mass.

Diffuse oxalate nephrocalcinosis without gross stone formation has been produced in cats by means of vitamin B<sub>6</sub> deficiency. This report concerns the counterpart of the phenomenon in rats. Weanling white rats maintained on pyridoxine-deficient diets for 5 to 10 weeks developed gross concretions at the tips of the renal papillas in 75 per cent of the animals (50 in 66) with mere traces of intrarenal crystallization. These concretions of relatively pure calcium oxalate monohydrate closely resembled human oxalate calculi. They were dislodged in life, frequently resulting in hydroureter (20 per cent of ureters) and occasionally in hydronephrosis or pyelonephritis. Small amounts of added dietary glycine appeared essential to the development of the calculi.

Alterations of the urine pH by addition of ammonium chloride or sodium bicarbonate to the drinking water altered the incidence and severity of lesions. Ammonium chloride produced urine of pH 5.7; sodium bicarbonate, of pH 7.7; while the control group, drinking distilled water, had urines of pH 6.7. Total renal oxalate deposits were more frequent in the group with acid urine (12 of 14 animals) than in the alkaline urine (7 of 14) or control (8 of 13) groups.

While the incidence of calculi still *in situ* on the papillas was similar for the 3 groups, they were largest in the acid urine group, and sequelae due to dislodgment were distinctly more severe. These animals demonstrated ureteral stone(s) with hydroureter in 10 of 28 instances, whereas in the alkaline urine and control groups this occurred in 3 of 28 and 1 of 26 rats respectively. Pyridoxine-supplemented controls exhibited no lesions of this nature.

**A URINARY BLADDER TUMOR INDUCED BY THE AGENT OF BOVINE CUTANEOUS PAPILLOMATOSIS.** C. Olson,\* M. Pamukcu, D. F. Brobst, E. J. Satter and J. M. Price, Cancer Research Hospital and University of Wisconsin, Madison, Wis.

A tumor of the urinary bladder was produced in 13 of 14 calves by the intramural injection of tissue from bovine cutaneous papillomatosis. The initial fibroblastic growth was accompanied by a polypoid reaction of the mucosa. Changes near the basement membranes were suggestive of early epithelial neoplasm. The pathologic features of the condition were somewhat similar to the epithelial hyperplasia and sarcoma-like reaction which occurred when the same provocative material was injected into the skin, causing warts, or when injected into the vaginal mucosa, causing fibropapilloma. The papilloma material also caused a sarcoma-like growth in the skin of the horse. These experimentally reproducible conditions occur naturally, although the tumor of the urinary bladder in cattle is restricted geographically to certain areas of India, Turkey, some European countries, and the Pacific northwest coast of the United States and Canada.

**UNSUSPECTED CARCINOMA OF THE PROSTATE IN SUPRAPUBIC PROSTATECTOMY SPECIMENS; A CLINICOPATHOLOGIC STUDY OF 55 CONSECUTIVE CASES.** Walter C. Bauer, Malcolm H. McGavran and M. Richard Carlin, Washington University School of Medicine, Barnes Hospital, St. Louis, Mo.

A long term follow-up investigation was made of 55 patients with unsuspected prostatic carcinoma. These were detected among 847 consecutive suprapubic prostatectomy specimens at Barnes Hospital. The importance of various factors in prognosis and treatment were evaluated. All 55 patients were followed for a minimum of 5 years: the crude 5-year survival rate was 54 per cent. Twenty-four patients were followed for 10 years, and their survival rate was 37 per cent.

Estimates of the degree of differentiation and size of the neoplasm were found to have a direct bearing upon the clinical outcome. The 5- and 10-year survival rates in patients with well-differentiated carcinoma were 75 per cent and 47 per cent, respectively. The 5- and 10-year survival rates for patients with poorly differentiated cancers were 33 per cent and 14 per cent, respectively.

The survival curve for patients with the small, well differentiated carcinomas was not demonstrably different from the life insurance survival curve for men of comparable age. Radical surgical procedures were not needed in this group of patients, nor was there any evidence that castration or estrogen therapy altered the prognosis.

On the other hand, patients with large or poorly differentiated carcinomas had a decidedly gloomy outlook and were not benefited by orchiectomy or estrogen therapy. On the clinical presumption that these patients were still operable, and in view of Jewett's results with cancers, "microscopically limited to the prostate," secondary radical prostatectomy is suggested as a rational therapeutic measure in these patients.

**PREVENTION OF HYPERTENSIVE VASCULAR DISEASE IN RATS GIVEN INTERMITTENT HYDRALAZINE.** D. L. Gardner, University of Edinburgh, Scotland, and Western Reserve University School of Medicine, Cleveland, Ohio.



Experiments were conducted with the object of reproducing the "hydralazine syndrome" in rats with steroid-induced hypertension, after unilateral nephrectomy had been performed. It was the practice to inject large amounts of hydralazine intramuscularly once daily. The dose given in this way ranged between 30 and 50 mg. per kg.; treatment was continued for 10 to 12 weeks. Under these circumstances, the systolic blood pressure was found to fluctuate widely once daily, dropping rapidly by as much as 100 mm. Hg during the first 3 hours after injection and returning slowly to the previous level within 24 hours. Comparable but smaller changes were recorded by direct manometry. Following such treatment, the remaining kidney, the heart, aorta, adrenals, mesentery and other tissues were examined histologically. By contrast with control animals which had also had unilateral nephrectomy but were untreated with hydralazine, there was almost complete avoidance of hypertensive vascular alteration.

These observations suggest that it is not essential to keep the mean blood pressure of hypertensive rats within the range of normality in order to prevent the evolution of vascular damage, and that large daily fluctuations in blood pressure do not necessarily result in hypertensive vascular lesions. In view of these observations, it does not appear possible to claim that vascular disease in rats with steroid-induced hypertension is the direct result of the rise in blood pressure alone. It appears more likely that the development of vascular alteration is mediated by a factor which is more sensitive to the influence of hydralazine than to the steroid pressor mechanism.

DIETARY-INDUCED CORONARY THROMBOSIS IN RATS: EFFECT OF VARIATIONS IN THE DIET.† Wilbur A. Thomas,\* Robert M. O'Neal,\* and W. Stanley Hartroft.\* Washington University School of Medicine, St. Louis, Mo.

Two years ago we reported the production of arterial thrombi and infarcts in rats by the administration of semi-synthetic diets supplemented by large amounts of fat and cholesterol and added propylthiouracil and bile salts. Since then, attempts have been made to elucidate the relative importance of these dietary constituents. We have performed an additional series of experiments involving more than 700 animals fed 49 variations of the original diets. Among rats receiving infarct-producing diets, the overall incidence of infarcts has been approximately 20 per cent in short term experiments (4 months). The incidence has varied widely (up to 60 per cent) in consecutive experiments even when the same food mixture was utilized. This made evaluation of dietary induced alterations difficult. However, certain features emerged:

(1) Production of infarcts in these animals was not shown to be dependent on inclusion of any single constituent of the basal diet (thiouracil; cholesterol; sodium cholate; saturated fat; 1 per cent choline chloride). (2) With corn oil, the incidence of infarcts was less, the level of plasma cholesterol lower, and the survival rate higher than with butter, lard or Crisco®. (3) Infarcts continued to appear although saturated fat was replaced by an excess of choline chloride (1.0 per cent). (4) Light and electron microscopy indicated that alterations in arterial walls were insufficient by themselves to account for thrombosis; i.e., plaques were not present although mural lipodosis was constant. (5) Alterations in clotting or fibrinolytic mechanisms were probably of considerable pathogenic importance, but more evidence is needed to support this concept.

ATHEROSCLEROSIS IN PIGEONS; ITS SPONTANEOUS OCCURRENCE AND RESEMBLANCE TO HUMAN ATHEROSCLEROSIS. T. B. Clarkson, R. W. Prichard,\* M. G.

† Supported by Grant H-1820 from the National Heart Institute and by the Nutrition Foundation.

Netsky and H. B. Lofland, Bowman Gray School of Medicine, Winston-Salem, N.C.

Certain breeds of pigeons (autosexing Kings, Silver Kings, and White Carneaux) were found to develop atherosclerosis spontaneously. The disorder was nearly identical with that in human beings. Another breed of pigeons (Show Racers), kept under the same conditions of diet, housing, and exercise, had almost no atherosclerosis. The diet consumed by these breeds was corn, wheat, peas and kaffir (milo).

The atherosclerosis involved the aorta, coronary arteries and, rarely, the cerebral arteries. It was characterized by raised, yellow plaques projecting into the lumen, with a predilection for the aortic bifurcation. Neutral fat and cholesterol crystals were present in the lesions. There was diffuse calcification in some lesions, and bone formation in others. The lipid and calcium were separated from the lumen by a thin layer of fibrous connective tissue in most instances. Occasionally there was ulceration and superimposed thrombus formation. The elastic tissue beneath the plaques was distorted.

Total, free, and ester serum cholesterol, and phospholipids were determined in all of the birds. There were no significant differences in serum lipid levels among the 4 breeds of pigeons despite marked differences in the severity of atherosclerosis. The weight of the aorta and its cholesterol content paralleled the severity of atherosclerosis.

**FATAL MYOCARDIAL INFARCTION IN THE RHESUS MONKEY WITH DIET-INDUCED HYPERCHOLESTEROLEMIA**† C. Bruce Taylor, George E. Cox, Marjorie Counts and Nelson Yogi, Presbyterian-St. Luke's Hospital, Chicago, Ill.

In earlier studies we reported the development of atheromas in the coronary arteries and the aorta and its major branches in Rhesus monkeys when mean cholesterol levels were maintained between 256 and 393 mg. per cent for 6 months or more. Hypercholesterolemia was induced by feeding an adequate diet containing 22 per cent butter fat and supplying 1.5 gm. of cholesterol per day. Two female animals on this diet showed a marked propensity for hypercholesterolemia. They were maintained on this diet for 3 and 4 years and had mean serum cholesterol levels of 679 and 554 mg. per cent. Both animals developed cutaneous xanthomas on the hands and feet. One animal died from massive myocardial infarction. Three thrombotic occlusions showing various stages of organization were found in the coronary arteries. The coronary arteries also showed marked athero-arteriosclerotic narrowing, medial degeneration with fibrosis, calcification or foam cell infiltration, markedly increased medial vascularization, and focal areas of secondary arteritis. The similarity to human coronary arteriosclerosis was striking. Arteriosclerotic lesions in the aorta and many other vessels also showed a striking similarity to human lesions.

**IMPLICATIONS OF GEOGRAPHIC DIFFERENCES IN THE SEVERITY OF ATHEROSCLEROSIS.** Ira Gore,\* William B. Robertson, Albert E. Hirst, G. Gordon Hadley\* and Yahei Koseki, Harvard Medical School and Harvard School of Public Health, Boston, Mass., University College Hospital, Jamaica, B.W.I., College of Medical Evangelists, Los Angeles and Loma Linda, Calif., and Sapporo Medical College, Sapporo, Japan.

By means of the assay procedure of Gore and Tejada, the aorta and coronary arteries from unselected patients examined at necropsy were quantitatively appraised for atherosclerosis. The study undertaken concurrently in Boston

† Supported by the Chicago, the Illinois, and the American Heart Associations, the Life Insurance Medical Research Fund, the Ames Co., Elkhart, Ind., and the Otho S. A. Sprague Memorial Institute.



and Los Angeles, U.S.A.; Jamaica, B.W.I.; Vellore, India; and Sapporo, Japan, included the following cases:

|                 | Aorta | Coronary<br>arteries | Myocardial infarcts<br>(40 and over) |
|-----------------|-------|----------------------|--------------------------------------|
| U.S.A.          | 1136  | 1168                 | 276 of 1073 cases                    |
| Jamaica         | 402   | 279                  | 6 of 217 cases                       |
| India (Vellore) | 258   | 77                   | 5 of 105 cases                       |
| Japan (Sapporo) | 260   | 258                  | 10 of 147 cases                      |

Myocardial infarction, an index of severe myocardial ischemia, was more than 3 times as frequent in the U.S. sample; the coronary atherosclerosis was most severe in this group as well. After the third decade, the progressive increase of atherosclerosis which occurred with aging was less marked in the coronary arteries than in the aorta. Lesser involvement of the coronary arterial tree, noted in previous studies of U.S.A. necropsy samples, was much more striking in the Indian, Japanese, and Jamaican groups. Aortic as well as coronary atherosclerosis was least advanced in India. In Japan the progressive increase of aortic atherosclerosis found after 30 years of age in the other locales, was delayed for about a decade; thereafter severity rose rapidly to equal that encountered in the later decades in Jamaica.

In general, both aortic and coronary atherosclerosis were more severe among the victims of myocardial infarction. Exceptions were frequent enough, however, to suggest that other factors than intimal disease *per se* entered into its occurrence.

**EFFECT OF PHYSICAL ACTIVITY ON EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN RABBITS.** Sidney D. Kobernick\* and G. Niwayama, Sinai Hospital, Detroit, Mich., and Children's Hospital, Buffalo, N.Y.

Three experiments were performed in which litters of rabbits, while being fed cholesterol, were trained to run in a treadmill. Half of each litter remained sedentary while the other half was permitted to exercise from 10 to 20 minutes per day. Ninety rabbits were used, and the amount of atherosclerosis observed in the aortas of the exercised animals was distinctly less than in the others. The decrease was not only evident by visual inspection, but was confirmed by chemical analysis. The serum lipids were also extensively investigated, and no correlation was observed between the level of the serum lipids, the total cholesterol to phospholipid ratio, and the degree of atherosclerosis. Exercise did not appear to produce a significant alteration of the serum lipid level.

The observations and possible pathogenesis of this phenomenon are discussed.

**INTERFEROMETRIC STUDIES IN ELASTIC FIBERS IN AORTAS OF ADULTS AND CHILDREN: MASS AND ALLIED PHENOMENA.**† Luiz C. Mattosinho Franca and Alvan G. Foraker,\* Baptist Memorial Hospital, Jacksonville, Fla.

The mass of elastic fibers has been compared in the aortas of adults and children in an introduction of interferometry to cardiovascular investigation. Unstained sections from 10 apparently normal aortas of infants and 10 of adults were examined with the 40X shearing system of the Baker-Smith microscope. Ten fields were examined in each case in deep, central, and superficial zones of the media. Optic path differences (in mounting media of refractive indices 1.490 and 1.515) were determined; the areas were photographed. Fiber size and concentration in a 2,500  $\mu^2$  sample were determined planimetrically.

† Supported by grants from the Damon Runyon Memorial Fund (DRG-352) and the National Cancer Institute (C-2719), National Institutes of Health.

The following were computed: refractive index, total particle thickness, dry mass per unit area, mean weight of a standard ( $50 \mu$ ) length of fiber, percentage area occupied by fiber in a  $2,500 \mu^2$  sample, and weight of fiber material in sample.

Statistical comparisons between observations in children and adults are in process and will be reported.

**THROMBOANGIITIS OBLITERANS. A CRITICAL RE-EVALUATION.** Si-Chun Ming, Stanford Wessler, Victor Gurewich, and David G. Freiman,\* Beth Israel Hospital and Harvard Medical School, Boston, Mass.

Among 1,464 Beth Israel Hospital patients with peripheral arterial obstruction, 84 had the onset of arterial insufficiency prior to age 45. A clinical diagnosis of thromboangiitis obliterans (TAO) was made in 48 of these patients; the remaining 36 had clinical evidence suggestive of atherosclerosis or arterial embolism.

Pathologic specimens from 26 of these 84 patients formed the study group and included 4 necropsies, 35 amputation specimens from 18 patients, and 14 superficial vein biopsy specimens from 14 patients. All 4 necropsies revealed evidence of multiple emboli. Two of these had healed myocardial infarcts with left ventricular mural thrombi; the other 2 hearts had multiple areas of myocardial fibrosis. Of the 18 patients with leg amputations, 10 showed atherosclerosis as well as thrombosis in the resected arteries; of the remaining 8 patients, 3 eventually showed evidence of multiple arterial emboli at necropsy. The organized thrombi in the arteries from the study group were indistinguishable from those observed in 66 injected limbs amputated for atherosclerotic gangrene and corresponded to the intermediate and healed arterial lesions described by Buerger. Many of the amputated specimens from the study group and from the 66 injected limbs also showed venous thrombi in various stages of organization, frequently in association with organized arterial thrombi and perivascular fibrosis. The acute "specific" arterial lesion described by Buerger was not found; its venous counterpart was found only in 2 superficial veins.

Review of the clinical and pathologic data suggests that the entity of TAO as described by Buerger produces a clinical and, in its intermediate and healed stages, a pathologic lesion which is indistinguishable from the alterations in patients with atherosclerosis or systemic arterial embolism. These data also suggest that the acute "specific" venous lesion is relatively uncommon and perhaps should not be accepted as definitive evidence of the existence of similar changes in arteries.

**NON-ATHEROMATOUS PERIPHERAL VASCULAR DISEASE OF THE LOWER EXTREMITY IN DIABETES MELLITUS.** Herman T. Blumenthal,\* Ram A. Joshi, Sidney Goldenberg, and Morris Alex, The Jewish Hospital, St. Louis, Mo.

A histochemical study of the small vessels of 152 amputation specimens of the lower extremity was carried out. Ninety-two of these specimens were in diabetic patients, and the remaining 60 in patients with a variety of other conditions necessitating amputation. A lesion of small arteries and arterioles was found in diabetic patients which differed from vascular lesions seen in the nondiabetic group. The vascular alterations in the diabetic series appeared to be analogous to those seen in diabetic nephropathy and retinopathy. They were characterized by endothelial proliferation and deposition of a webwork of a PAS-positive, colloidal iron-negative material. They could be distinguished from arterio- and arteriolar sclerosis.

Such vascular lesions were found in the vasa vasorum and peri-adventitial vessels of cognate systemic arteries, as well as in the small arteries and

arterioles of nerves, muscles and skin. They were found in diabetic patients without evidence of hypertension and in some who had not received insulin prior to amputation. Clinical correlations were carried out with respect to the manifestations of neuropathy, retinopathy and nephropathy, as well as to pulse characteristics and the distribution of gangrene.

**PATHOLOGIC LESIONS OF THE HUMAN CARDIAC CONDUCTION TISSUE.** George Lumb,\* R. S. Shacklett and L. B. Otken, Jr., University of Tennessee, Memphis, Tenn.

In order to study the atrioventricular (AV) node and the Bundle of His and its branches in the human subject, the interventricular septum was removed from 250 hearts. The block of muscle was limited posteriorly by the entrance of the coronary sinus into the right atrium and anteriorly by the papillary muscle of the conus. The sides were formed by the septal walls of the ventricles. This area of muscle was divided into 3 or 4 equal blocks according to the size of the heart. In the most posterior sections the AV node was seen to the right and above the annulus fibrosus. As sections were traced anteriorly, the course of the bundle and its branches could be defined.

Serial sections were not found to be necessary, once the structure was recognized. All age groups were studied in the series. The normal characteristics of the tissue were first established and then pathologic alterations were correlated with abnormalities noted before death.

In 26 cases electrocardiographic changes were observed indicating conduction defects. Nineteen of these showed pathologic lesions. Fourteen hearts showed damage of the AV node, the bundle or its branches, but no electrocardiographs had been made. Six of these were examples of sudden death, and in 10 cases, including the 6 above, cardiac failure was considered to be the immediate cause of death. The lesions found to be causing the damage to the conduction tissue were grouped as: ischemia, including infarction, fibrosis and calcification; inflammation, including myocarditis, abscesses and rheumatic fever; hemorrhage; amyloidosis; and fibroelastosis.

**THE *In Situ* ABSORPTION SPECTRUMS OF HYALIN, FIBRINOID AND AMYLOID SUBSTANCES.**† Boris Gueft,\* Albert Einstein College of Medicine, New York, N.Y.

Ultraviolet and visible absorption spectrums of these substances were obtained with an automatic recording microspectrophotometer. The hyaline material of arteriosclerosis showed slight 2,800 Å absorption. This absorption in tissue sections is not found in collagen normally. It is usually ascribed to tyrosine, tryptophan and other amino acids normally found in other proteins.

The Kimmelstiel-Wilson hyalin had similar absorption. The fibrinoid masses found in necrotizing polyarteritis showed moderate absorption at 2,800 Å, consistent with increased protein content. The fibrinoid substance of necrotic arterioles in malignant nephrosclerosis showed only 2,800 Å absorption of moderate degree. Derivation of this fibrinoid from necrotic smooth muscle has been postulated. If this is so, myoglobin might be detected by its intense Soret absorption band at about 4,150 Å. No such band was found in formalin-fixed, paraffin-embedded unstained sections. The possibility remains that myoglobin leaves the necrotic zone either *in vivo* or during preparation of the specimen. The fibrinoid substance in the Libman-Sacks vegetation in systemic lupus had marked absorption at about 2,700 Å. This may be interpreted as indicating the presence of purine or pyrimidine compounds derived from the nucleoprotein

† Aided by grants from the Veterans Administration and the United States Public Health Service (A-2967).

deposited by the LE phenomenon. The absorption spectrums of secondary, atypical, cardiac, and "myeloma" amyloids all showed pronounced 2,800 Å absorption. The dense connective tissue of scleroderma had a weak 2,800 Å absorption. No visual absorption bands were noted in any of the above lesions.

A CLINICOPATHOLOGIC ANALYSIS OF "RHEUMATOID" NODULES OCCURRING IN 54 CHILDREN. J. H. Draheim, L. C. Johnson, and E. B. Helwig.\* Armed Forces Institute of Pathology, Washington, D. C.

Subcutaneous nodules histologically resembling those of rheumatoid arthritis and rheumatic fever occurred in a group of 54 children without the stigmas of rheumatic disease. These were characterized by foci of necrobiosis, marginal palisading of spindle shaped cells, an associated chronic inflammatory exudate, and vascular proliferation. It was considered important to establish the significance of the lesion in respect to the possibility of future rheumatic disease in these patients.

The majority of the nodules occurred in children 3 to 6 years of age. Follow-up for periods of 1 to 14 years showed that only an occasional patient developed rheumatic fever and none developed rheumatoid arthritis. In about a fourth of the patients, trauma appeared to be the only significant accompanying feature. Superficial skin involvement suggesting granuloma annulare was noted in approximately the same number of children, and that clinical diagnosis was made in several instances. Allergy was present in a sufficient number of cases to suggest a relationship.

We believe that this histologic lesion occurring in the subcutaneous tissue is not a specific reaction pattern, and that the diagnosis of rheumatic disease based solely on its presence is not warranted.

## SYMPOSIUM ON CONDITIONING FACTORS IN NEOPLASIA

Referee (by invitation of the Council): Jacob Furth

A CONCEPT OF TUMOR DEPENDENCY AND AUTONOMY. Harry S. Greene,\* Yale University, New Haven, Conn.

Abstract not received.

STUDIES OF THE AMINO ACID METABOLISM OF A VARIETY OF TUMOR SORTS. Stanfield Rogers,\* The University of Tennessee Memorial Research Center, Knoxville, Tenn.

The effect of hormones on tumors arising in tissue influenced by them has been long known and has pointed up the critical importance of the physiologic state of the animal upon tumor growth. Work in this laboratory has been concerned with the amino acid metabolism of individual tumors, and a wide variety have been studied. The variation in amino acid utilization from tumor to tumor, even among those of the same morphologic class, has been large. In addition to providing another means of classifying tumors, the information provided some knowledge of the metabolic requirements of cells and thereby cast light upon physiologic conditions which, like the hormones, probably are critical conditioning factors in the maintenance of the cells as tumor cells. When hormones were added to the media in which hormone-dependent cells were being incubated, the effect provided information as to the metabolic means of operation of the individual hormones as well.

PROGRESSION IN TRANSPLANTABLE THYROID TUMORS ARISING IN RATS FED THIOURACIL. Seymour H. Wollman and Edward B. Price, Jr., National Cancer Institute, Bethesda, Md.

Transplantable thyroid tumors were produced in inbred Fischer rats by chronic feeding of 0.25 per cent thiouracil followed by transplantation of hyperplastic thyroid tissue. Early nodules observed were papillary. Later, lesions were characterized by diffuse cellularity, microfollicular structure, or transition stages between these. These patterns were found in tumors which grew only in conditioned rats (dependent tumors).

After several transplantation generations, implants began to grow in rats which were not conditioned (independent tumors). The histologic patterns seen in these independent tumors occasionally resembled those of the parent dependent tumors, but sometimes were markedly different. A variety of histologic types were characterized by variations in cell type, follicular structure, and the presence of colloid or mucin. Staining properties of the colloid and specific types of inflammatory cells in the lumens of follicles were also varied. Progression of histologic types and of radioiodine metabolism were studied.

First generation independent tumors took approximately one year to become palpable whereas later generations took a month or two. This suggested that the independent tumors arose from a relatively small fraction of the implant. Hypophysectomy decreased the growth rate of almost all independent tumor lines, even the most anaplastic. Thiouracil feeding rarely stimulated growth of tumors which were independent for more than a few generations.

TRANSPLANTABLE THYROID TUMORS INDUCED BY THYROTROPIC PITUITARY GRAFTS. Peter Pullar and Nechama Haran-Ghera, Children's Cancer Research Foundation, Boston, Mass.

A transplantable thyroid tumor strain has been developed in LAF<sub>1</sub> mice by prolonged stimulation of the glands with thyrotropic hormone. The source of the latter was an autonomous but highly functional thyrotropic pituitary

tumor which had been grafted intramuscularly in the animals. Under its influence the thyroid became greatly enlarged and nodular, the histologic appearance being that of adenoma with possibly some areas of adenocarcinoma. The thyroid lesions were transferable as intramuscular grafts to other animals in which they developed as slowly growing tumors. Transplantation was only successful in hosts which also had a grafted functional thyrotropic pituitary tumor. Histologically, the appearance of the grafted thyroid tumors ranged from papillary adenoma to well differentiated adenocarcinoma.

**STUDIES ON THE DEPENDENCY OF THYROID CANCER.** Colin G. Thomas, Jr., University of North Carolina School of Medicine, Chapel Hill, N.C.

The production of thyroid neoplasms in the experimental animal has demonstrated the promoting effects of thyrotropic hormone and the evolutionary nature of such tumors. Although conditions preceding the development of thyroid neoplasms in man are not so conclusive, there is suggestive evidence that their evolution is enhanced by a thyrotropic hormone stimulus. During the past 4 years, 15 patients have been studied in an effort to evaluate the role of thyrotropin in the growth progression and function of human thyroid cancer. Parameters of measurement following alterations in thyrotropic hormone levels induced by hypothyroidism or factitious hyperthyroidism have included the gross and microscopic appearance of the neoplasm, uptake of radioiodine, output of thyroid hormone (as protein-bound iodine), and uptake of radiophosphorus. Experience during this time has disclosed that certain types of thyroid cancer exhibit a striking dependence upon thyrotropic hormone as demonstrated by alterations in growth, function, and decrease in metabolic activity. Other tumors are completely independent, and still others, while initially dependent, may exhibit increasing autonomy.

**CONDITIONING FACTORS IN HUMAN CARCINOMA OF THE THYROID.** John B. Hazard,\* Cleveland Clinic, Cleveland, Ohio.

Conditioning factors may influence the incidence of thyroid carcinoma or alter its growth propensity after establishment. The investigation was based on a series of approximately 300 carcinomas encountered since 1945.

There was an age group of greater incidence for certain types of carcinoma; for anaplastic carcinoma the peak appeared in the seventh decade, whereas in papillary carcinoma it was in the fourth. In the series presented, papillary carcinoma was practically the exclusive type of thyroid carcinoma in childhood. The preponderant occurrence of carcinoma of the thyroid in females is well recognized, particularly the papillary type.

Nodular goiter has been recognized in the past as an important conditioning factor in the origin of carcinoma. In this series, however, approximately 70 per cent of papillary carcinomas occurred in glands without nodular alteration. Struma lymphomatosa has been reported to occur in carcinoma with appreciable frequency but in the present series did not exceed 5 per cent for any type of carcinoma.

Radiation to the head, neck or thorax has been regarded by some as a factor in the occurrence of carcinoma in children. Eleven of 14 children with carcinoma of the thyroid had a history of radiation to such areas when they were infants. No instance of previous therapy with radioiodine was encountered although there were several examples of papillary carcinoma occurring in the thyroids of patients who had received a thiouracil compound.

Treatment of thyroid carcinoma with thyroid hormone, usually in the form of desiccated thyroid, appeared to halt or moderate the disease in certain patients with papillary carcinoma and in a few with follicular carcinoma,



but did not seem to be effective in the anaplastic type. Rarely, a transition from the papillary type of carcinoma to a mixed type including anaplastic forms was encountered after radiation; because of the paucity of cases, the significance of this cannot be completely evaluated. Recurrence of papillary carcinoma at the sites of surgical excision, though not altering the architectural type, appeared to be a factor in increasing the lethal effect of the tumor both regarding local regrowth and potentiality for metastasis.

**AN INVESTIGATION OF THE EFFECT OF HYPOPHYSECTOMY ON THE COURSE OF CARCINOMA OF THE BREAST.**† Alvin Volkman, Diane W. Crocker, Andrew Jessiman and Kendall Emerson, Jr., Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.

Following hypophysectomy, some patients with carcinoma of the breast exhibit objective as well as the subjective improvement. The former is observable as gross diminution in size of the primary tumor and of metastases. A study has been undertaken to seek possible histologic evidence of alterations in the structure of malignant cells that may have resulted from hypophysectomy.

The group selected was comprised of 10 women who had survived for a sufficient period of time after hypophysectomy to permit one, and in certain cases, serial biopsy examinations of neoplastic tissue. Tumor tissue removed prior to hypophysectomy was used as a baseline. In addition, necropsy material was examined.

A parallel study of endocrinologic and metabolic alterations was carried out on some patients. An attempt was made to correlate these observations with histologic evidence of alteration of tumor.

Two patients who had hypophysectomy performed during pregnancy were studied in detail from both aspects. A comparison is made with patients with carcinoma of the breast in whom hypophysectomy was not performed.

**INDUCTION AND TIME OF APPEARANCE OF BREAST NEOPLASM IN THE RAT FOLLOWING WHOLE BODY RADIATION.**‡ C. J. Shellabarger, S. W. Lippincott,\* E. P. Cronkite, and V. P. Bond. Brookhaven National Laboratory, Upton, N.Y.

A linear response has been reported in the occurrence of neoplasm of the breast when female Sprague-Dawley rats were examined 11 months after a single exposure to whole body radiation in the range of 25 r. to 400 r. In the present experiments, groups of 28 or 30 40-day-old female Sprague-Dawley rats were studied from the day of exposure to 200 r. or 400 r. of  $\text{Co}^{60}$  gamma radiation until a histologically verified neoplasm of the breast was found or until death. Thirty litter mate rats served as nonexposed controls. The incidence of neoplasm of the breast in rats was: controls, 37 per cent; 200 r., 56 per cent; 400 r., 90 per cent. The first breast neoplasm occurred approximately 60 days after radiation. At all periods thereafter, 400 r. produced a tumor yield that was approximately twice that in the 200 r. group. In both exposed groups, at all times, the incidence was greater than in the nonexposed group. It would appear that a single exposure to ionizing radiation can result in (a) hastening the onset of a neoplastic process which occurs spontaneously in nonexposed animals, and (b) inducing more neoplasms than would occur in nonexposed rats of the same age and allowed to live out their life span. It is concluded that these data do not lend support to the often expressed idea that neoplasm

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‡ Supported by the United States Atomic Energy Commission.



following exposure to radiation results from an "acceleration of the aging process."

**HISTOLOGIC STUDY OF AUTONOMOUS AND HORMONE-SENSITIVE TUMORS OF THE BREAST AND PROSTATE.** Victor Rosen and Edgar B. Taft,\* Massachusetts General Hospital, Boston, Mass.

Some years ago an attempt was made to confirm the suggestions made by Huggins and Dao that response to adrenalectomy and oophorectomy of patients with far advanced carcinoma of the breast could be correlated with the histologic pattern of the tumors. Since this was unsuccessful in a small series of cases, no further observations were carried out at that time. Recently, stimulated by some remarkable remissions in tumors of the prostate after hormonal therapy and by suggestions that correlation was possible between histologic structure and autonomy in tumors in experimental animals, we studied two further groups of patients with carcinoma of the breast and carcinoma of prostate. These patients fell into two groups: those who responded well clinically to hormonal therapy and those who did not respond at all. The histologic findings in these two groups are presented and discussed.

**HORMONE RESPONSIVE AND NON-RESPONSIVE MAMMARY GLAND AND PROSTATIC TUMORS.** Eleanor Humphreys,\* University of Chicago, Chicago, Ill.

Abstract not received.

**LAG IN APPLICATION OF EXPERIMENTAL STUDIES ON CONDITIONED AND AUTONOMOUS NEOPLASMS IN HUMAN TUMOR-PATHOLOGY.** Jacob Furth\* (Referee†), Children's Cancer Research Foundation, Inc.

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**PATHOLOGY OF EXPERIMENTAL PULMONARY TUBERCULOSIS ADEQUATELY TREATED; PRODUCTION AND RESOLUTION OF PULMONARY CYSTS.** Raymond Yesner,\* Sidney Bernstein, and Nicholas D'Esopo. Veterans Administration Hospital, West Haven, Conn.

Ninety-six rabbits were exposed to infective aerosols containing bovine tubercle bacilli. At 6 to 7 weeks, they were found by roentgen examination, and in several animals by necropsy, to have extensive primary pulmonary tuberculosis without cavity or cyst formation. The remaining rabbits were treated with 100 mg. of streptomycin and 24 mg. isoniazid by daily intramuscular injection for one year. Those animals which died or were sacrificed during the first 2 months of therapy showed formation of numerous, massive multiloculated, thin-walled bullas, to which clung masses of necrotic, calcified debris, and macrophages. Thin septums projecting into the cavities contained central bronchioles. No large bronchi could be found entering any bulla. These data suggested that the early explosive appearance of the bullous lesions was the result of adequate drug therapy. The fact that the bullas projected above the surface without collapse indicated an air-trapping mechanism, possibly associated with the septal bronchioles. The animals which died during the next 10 months of therapy showed evidence of rapid and extensive resolution of the bullous lesions, which were converted into collapsed, small, stellate structures, still containing a small number of macrophages. At the end of one year of therapy, 20 per cent of the surviving rabbits showed persistence of a small number of inflated bullas; 80 per cent were practically restored to normal.

† By special invitation of the Council.

Six weeks after infection, the lungs in these animals were filled with caseous masses; 30 days after therapy was instituted, the lungs contained a congeries of cysts and resembled polycystic kidneys.

**RESISTANCE OF TUBERCULIN SENSITIZED MONOCYTES TO INTRACELLULAR TUBERCLE BACILLI.** Morgan Berthrong,\* Glockner-Penrose Hospital, Colorado Springs, Colo.

When cells from animals which have been sensitized by injections of bacteria or combinations of certain bacterial products and adjuvants are placed in tissue culture with adequate concentrations of proteins from the bacteria, the cells are usually damaged or killed though normal cells are not injured. Most such studies have been performed with cells sensitized to tubercle bacilli and tested with OT or PPD. During tissue culture studies to investigate the resistance of normal and immunized guinea pig monocytes to virulent, multiplying, intracellular tubercle bacilli, a remarkable resistance of the immunized and sensitized monocytes to large numbers of intracellular bacilli was observed. Initially, the death rate of hypersensitive monocytes was slightly greater during the first 48 hours. Thereafter, however, these cells were at least as capable of withstanding the effects of living or killed intracellular tubercle bacilli as normal monocytes. Indeed, completely normal structure and migration were usually observed in monocytes from animals which responded to OT with necrotic skin reactions in spite of the presence within a cell of 50 or more intact tubercle bacilli. The same cells were injured by OT, however, under identical cultural conditions. Sensitized monocytes, infected *in vivo* by the intravenous injection of tubercle bacilli, migrated from the splenic fragments in tissue culture, as well as similarly infected nonsensitized monocytes.

Tissue necrosis in the tuberculous host, therefore, may be a consequence of bacillary death and the reaction of sensitized cells to protein derivatives rather than intact bacilli. Tissue culture studies using monocytes from animals sensitized (and immunized) by airborne infection, by BCG vaccination, and by injections of killed tubercle bacilli in mineral oil are presented. *In vitro* infections with virulent and attenuated, living and killed tubercle bacilli have also been employed.

**HISTIOCYTIC GRANULOMATOUS MYCOBACTERIAL LESIONS PRODUCED IN THE GOLDEN HAMSTER (*Cricetus auratus*) INOCULATED WITH HUMAN LEPROSY.** Chapman H. Binford,\* Armed Forces Institute of Pathology, Washington, D.C.

During the past 3 years, more than 35 experiments have been initiated in the attempt to infect animals with human leprosy. Monkeys, guinea pigs, albino hamsters, golden hamsters, white rats, white mice and hairless mice have been used. Resistance-reducing techniques included total body irradiation or cortisone. Inoculations were usually made into anatomic sites where the normal temperature of the tissue was relatively low. All experiments were controlled by identical inoculation of heat-killed human leprosy bacilli.

In two experiments with the golden hamster (*Cricetus auratus*), inoculations were made into the ears and testes. Histiocytic granulomas containing intracellular acid-fast bacilli appeared in these sites in some animals. The lesions were localized to these regions. A period of approximately one and a half years was required for the histiocytes to become filled with acid-fast bacilli. An important feature of the lesions of the ears was the presence of large numbers of bacilli within nerves. The effect of cortisone could not be appraised because all of the cortisone treated animals died of secondary infections within a few months. There was no significant difference in the lesions in the irradiated group and those in hamsters which had not been irradiated.

After 4 months' observation, the first transfer experiment, using hamster testis to hamster testis, appeared successful. A slowly growing acid-fast bacillus was obtained in Löwenstein's medium, but investigations of this bacillus have not been completed.

While this intracellular mycobacterial granulomatous infection resembles lepromatous leprosy histologically and the invasion of nerves is a characteristic of human leprosy, no conclusions as to the identity of the etiologic agent can be made at this time.

In none of the many experiments with other animals was any evidence of mycobacterial growth obtained.

**LOCALIZATION OF ALLERGIC ENCEPHALOMYELITIS IN LESIONS OF CYANIDE ENCEPHALOPATHY.** Seymour Levine,\* St. Francis Hospital, Jersey City, N. J.

Experimental allergic encephalomyelitis was produced in rats by injections of nervous tissue with Freund's adjuvant. It was characterized by prominent cellular proliferation in and around the walls of blood vessels with variable degrees of perivascular demyelination and with greater involvement of spinal cord, medulla and cerebellum than forebrain. Cyanide encephalopathy was produced in rats by a single exposure to hydrogen cyanide. It was characterized by extensive demyelination with a vascular reaction that was clearly secondary and with specific localization in certain forebrain structures (corpus callosum, corpus striatum, etc.).

This study was based on a series of rats, each of which had histologically proven lesions of both types. The cyanide lesions were produced at various times before and during the incubation period of allergic encephalomyelitis and also after signs of paralysis had developed. The distribution of cyanide lesions in these animals was the same as that seen in controls. In contrast, the distribution of allergic encephalomyelitis lesions was altered due to localization in and around cyanide lesions. The tendency to localization was noted in rats killed as early as one day after cyanide exposure, indicating that the lesions of allergic encephalomyelitis developed rapidly. The tendency to localization was decreased when the cyanide lesions were several weeks or months old. The combined lesions were characterized by parenchymal and vascular reactions which were more intense than those seen in either condition separately. Cyanide lesions were associated with local increase of permeability to trypan blue, suggesting that increased penetration of a noxious agent may have been responsible for localization of allergic encephalomyelitis.

**COMPARISON OF FLUORESCENT ANTIBODY STAINING AND SPECIAL HISTOLOGIC STAINS FOR THE IDENTIFICATION OF *Cryptococcus neoformans*.** John D. Marshall, Jr., Lalla Iverson,\* Warren C. Eveland and Alice Kase, Armed Forces Institute of Pathology, Washington, D.C.

Certain types of tissue response to *Cryptococcus neoformans* are difficult to recognize specifically unless the organisms can be identified with the mucicarmine stain. Notably, these are: non-caseating granulomas resembling histoplasmosis, sarcoidosis, or tuberculosis; fibrocaceous coin lesions of the lung resembling coccidioidomycosis, histoplasmosis, or tuberculosis; proliferating macrophagic reactions in lung or bone; and healing lesions. In some sections, few organisms are present and may be missed; in others, such as the fibrocaceous lesions, the capsular substance may be sparse and only faintly stained with mucicarmine stain. In these lesions it was desirable to determine the practical value of the fluorescent labeled antibody technique of Coons and co-workers as a diagnostic adjunct; and in such difficult tissue patterns to compare the results of the

specific fluorescent staining with the simpler, more readily available methods of ordinary microscopy. Serial sections from numerous selected lesions were stained by hematoxylin and eosin, mucicarmine, Gomori methenamine silver stains, and by the fluorescent technique. In general, the fluorescent staining revealed the capsules of the organisms more sharply than did the mucicarmine stains; but the numbers of organisms stained and the variations in staining intensity were similar in both preparations. In some instances, where no intact organisms could be observed, deposits of capsular polysaccharide could be identified with the fluorescent antibody technique.

**THE DEMONSTRATION OF GAMMA GLOBULIN IN LIVERS WITH POSTNECROTIC CIRRHOSIS AND FULMINATING VIRAL HEPATITIS, USING THE FLUORESCENT ANTIBODY TECHNIQUE.** Seymour Cohen, Goroku Ohta, Edward J. Singer, Richard E. Rosenfield, Ely Perlman and Hans Popper,\* The Mount Sinai Hospital, New York, N.Y.

In continuation of studies dealing with the cytoplasmic basophilia and the capacity to form protein in mesenchymal cells of the liver, spleen and lymph nodes in hepatic diseases, gamma globulin was demonstrated in these cells by the Coons fluorescent antibody technique. Livers from patients with inflammatory and other disorders, various types of cirrhosis and fulminating viral hepatitis were examined.

In normal livers, even in the case of hypergammaglobulinemia unassociated with hepatic disease, cells containing gamma globulin were virtually absent. This substance was present in many cells in fulminating viral hepatitis and in a moderate number in postnecrotic cirrhosis. Few cells contained gamma globulin in diffuse septal, biliary, or cardiac cirrhosis. Gamma globulin was found mainly in oval, basophilic, plasmocytoid cells (apparently mobilized Kupffer cells). In several stages in the apparent transition of plasmocytoid cells from Kupffer cells, these elements were seen to contain gamma globulin. In the fibrous septums, similar oval, basophilic, plasmocytoid cells as well as histiocytes and plasma cells contained gamma globulin. Here again the substance was noted in cells transitional from histiocytes to plasmocytoid cells. Occasionally histiocytes and, less frequently, Kupffer cells containing gamma globulin also exhibited PAS-positive staining or lipofuscin. Accumulations of lymphoid cells, particularly in biliary cirrhosis, were free of gamma globulin.

Studies are in progress to investigate whether the gamma globulin found in mesenchymal cells in the conditions described represents in part an antibody against hepatic tissue. The possible role of the PAS-positive material in these cells in stimulating the formation of gamma globulin is also under investigation.

**INCLUSION-BEARING CELLS IN THE URINE DURING MEASLES AND OTHER ACUTE VIRAL INFECTIONS.** Robert P. Bolande,\* Western Reserve University, Cleveland, Ohio.

Papanicolaou stains of the urinary sediment during acute exanthematous and enanthematous viral infections in children revealed the presence of peculiar inclusion-bearing cells. The typical cell varied from 15 to 40  $\mu$  in diameter and was roughly circular, with abundant finely granular cytoplasm. The nucleus was pyknotic and displaced to the periphery of the cell by the intracytoplasmic inclusion. The inclusion was round and sharply demarcated from the surrounding cytoplasm. It tended to be brightly eosinophilic and refractile. Less commonly, the inclusions appeared club-shaped and, on occasion, were fragmented. The inclusion-bearing cells were present in greatest number and in their best developed form during the late prodrome and early rash stage of measles. They

were identified in 16 of 20 cases of measles on the initial examination of the urine. Similar cells were also present in German measles, mumps, chicken pox, and herpangina. The origin of the cell was uncertain, but it was thought to be a monocytic element possibly derived from the blood or urinary tract stroma. It is possible that the inclusions are the cytopathic effects of viral infection of these cells. More critical and direct investigation must be undertaken to establish the significance of these observations.

**ELECTRON MICROSCOPY OF NUCLEAR INCLUSIONS.**† Sarah Luse,\* Jack Davies, and Sam L. Clark, Jr. Washington University School of Medicine, St. Louis, Mo.

Although cytoplasmic ingestion of colloidal materials by pinocytosis has been thoroughly documented, nuclear penetrability has been relatively ignored. Electron microscopy demonstrated the existence of nuclear pores and the continuity of the outer nuclear membrane and ergastoplasmic membranes. Our observations suggest that not only pores but also pinocytosis by the nuclear membrane are involved in nuclear penetration. Intranuclear lipid inclusions occurred in normal fetal liver and mouse yolk sac endoderm. Choline deficiency or intravenous cod liver oil led to nuclear lipid, and with both, inclusions were often surrounded by inward extensions of nuclear membrane. Cytoplasmic lipid sometimes indented the nucleus.

Nuclei of newborn rat duodenum examined prior to feeding were free of inclusions, but after suckling, numerous dense nuclear inclusions appeared. Injection of either colloidal carbon or saccharated iron led to nuclear inclusions in rabbit yolk sac. Nuclear inclusions composed of viral particles developed in cells infected by several viruses. In view of the ability of particulate and lipoprotein materials to enter the nucleus, nuclear penetration by virus may be postulated to occur by either nuclear pinocytosis or through pores. Nuclear permeability is discussed in relation to immunity.

**ELECTRON MICROSCOPE OBSERVATIONS OF THE EPIDERMAL MELANOCYTE, FOLLOWING A MODIFIED DOPA REACTION.** Wallace H. Clark, Jr.,\* Ben E. M. Watson, and Mildred Watson, Tulane University School of Medicine, New Orleans, La.

The dopa positive cells in human epidermis are thought to be the site of melanogenesis and are probably the cells of origin of nevi and melanomas. These cells, the melanocytes, are believed by some to comprise a distinct cellular system within the epidermis. Others feel that they are only a variant of ordinary basal cells. Electron microscope studies have shown that the epidermis contained cells that were distinguished easily from the ordinary epidermal cells. Located chiefly in the basal layer, these had cytoplasmic processes (dendrites) that extended between adjacent epidermal cells. If these cells are identical with the dopa positive melanocytes observed with the light microscope, they too should be dopa positive. We have modified the dopa reaction for electron microscopy. Tissues were fixed for one hour in veronal acetate-buffered one per cent formalin. They were incubated for 7 hours in a buffered dopa solution; followed by fixation for 2 hours in Palade's veronal acetate-buffered osmic acid. The epidermis, incubated in dopa, showed dendritic cells with an increased number and size of melanin granules and a diffuse increase in electron density of the cytoplasm. Dendritic cells in control tissues did not show these changes. Ordinary epidermal cells were not altered, indicating that in normal epidermis only dendritic cells are dopa positive. These observations go far toward establishing the identity of the dendritic cell seen in electron micrographs with the melanocyte described by light microscopists.

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The observations suggest that the melanocytes are a distinct cellular system within the epidermis.

**HISTOLOGIC CHANGES IN THE HUMAN SEBACEOUS GLAND FOLLOWING THE ADMINISTRATION OF ACTH AND HYDROCORTISONE.** John S. Strauss, Boston University School of Medicine, Boston, Mass.

Either ACTH or hydrocortisone was given to 6 prepuberal males, 4 postpuberal men, and 6 postpuberal women. Control cutaneous biopsy specimens were procured from the cheek, and after a variable period of treatment a second specimen was taken from the same position on the contralateral cheek. All specimens were sectioned serially and stained with hematoxylin and eosin. Two of the 3 prepuberal subjects receiving 80 units of ACTH gel 3 times a week showed definite sebaceous gland hyperplasia; 2 of the 3 prepuberal males receiving 60 mg. of Cortef® per day showed definite sebaceous gland hyperplasia. Similarly, 2 of the 3 women receiving hydrocortisone developed sebaceous gland hyperplasia. The 4 postpuberal men did not show any change in the sebaceous glands.

In general, the alterations following ACTH administration were greater than those produced with hydrocortisone, an observation which can be correlated with the more common occurrence of acne after ACTH administration as compared to hydrocortisone. The induction of sebaceous gland hyperplasia by these two hormones may be of importance in the causation of normal glandular enlargement in females at the time of puberty.

**THE IDENTIFICATION OF NUCLEAR RIBOSE NUCLEIC ACID WITH GRAM'S STAIN.** Marvin Murray, University of Wisconsin Medical School, Madison, Wis.

Previous investigation has indicated that the fraction of the bacterial cell staining with crystal violet in Gram's stain is ribose nucleoprotein. This technique has not been applied to the staining of mammalian cells because of the variability in staining fixed tissues. In the present study, fresh tissues were cut by the frozen section technique. Tissue imprints and smears were also made. All were heat fixed. Under standardized conditions, all cells stained similarly with the Gram stain. The cytoplasm was light pink; the nucleus was red; and substance in the nucleolar area took the crystal violet stain. No purple staining material was noted in the cytoplasm of any cell. Control sections stained for RNA material with pyronin methyl green indicated the presence of this substance in similar areas in the nucleus as well as in the cytoplasm.

Sections and smears were incubated at 37° C. for 24 hours with ribonuclease in borate buffer (pH 7.4). Tissues thus treated and stained contained no substance which would accept the crystal violet stain. Areas containing no stain were noted in the cytoplasm as well as in the region of the nucleolus. Similar results were noted with the pyronin methyl green stain. Precipitated commercial RNA stained purple with Gram's stain, whereas precipitated DNA stained red.

These results suggest that crystal violet under these conditions stains nuclear RNA substance specifically. This would indicate a chemical difference between nuclear and cytoplasmic RNA.

**EFFECT OF  $\beta$ -AMINOPROPIONITRIL ON EXTRACTABILITY OF COLLAGEN FROM SKIN OF MATURE GUINEA PIGS.**† Jerome Gross and C. I. Levene, Massachusetts General Hospital and Harvard Medical School, Boston, Mass.

It was shown previously that in guinea pigs short periods of weight loss

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incident to caloric restriction greatly diminished the yield of collagen extractable from the skin with cold neutral salt solutions (0.45 M NaCl). This fraction was also absent from the skin of aging guinea pigs. Parenteral or oral administration of  $\beta$ -aminopropionitril daily for a period of 2 to 3 weeks to mature (400 to 800 gm.) and aging (greater than 1,000 gm., 1 to 2 years old) guinea pigs resulted in steady weight loss but a considerable increase in the amount of collagen extractable from the skin in cold neutral salt solutions. The same occurred with the administration of this agent to partially starved animals. Thus, the lathyrogenic agent induced the appearance of a collagen fraction under conditions of inanition and aging which ordinarily caused its reduction to negligible amounts. This phenomenon may represent new, normal, or abnormal synthesis, or transformation of old insoluble collagen to a soluble state.

**THE DIRECT HISTOCHEMICAL DEMONSTRATION OF DPNH AND TPNH DIAPHORASES AND ITS APPLICATION TO HISTOLOGIC PROBLEMS.** Richard B. Cohen, Massachusetts General Hospital, Boston, Mass.

Histochemical methods utilizing reduced coenzymes DPNH and TPNH as substrates for the demonstration of their respective diaphorases were applied to histologic problems. The methods depend on the deposition of the reduced form of Nitro-BT, [2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-ditetrazolium chloride], at the site of enzyme activity. This technique was utilized in combination with the histochemical methods for the DPN and TPN dehydrogenase systems to make these reactions more informative. The latter techniques depend on the action of at least two enzymes, a dehydrogenase which catalyzes the transfer of electrons from an oxidized substrate to the coenzyme, and a diaphorase which catalyzes the transfer of electrons from the coenzyme to the Nitro-BT, reducing it. The resultant deposition of dye localizes diaphorase activity only in those areas in which both dehydrogenase and diaphorase are present. If no reaction is obtained, a defect may exist in either enzyme. The methods utilizing DPNH or TPNH as substrates do not depend on dehydrogenase activity but demonstrate general tissue diaphorase directly. The direct technique, when utilized in combination with the dehydrogenase method, excludes or confirms the presence of a defect in the diaphorase portion of the system. Applications of the techniques to histology are described. The distribution of the enzymes in epithelial, muscle and nervous tissues provides interesting histologic data as well as biochemical correlations.

**RAPID IDENTIFICATION OF MALIGNANT CELLS IN VAGINAL SMEARS BY FLUORESCENCE MICROSCOPY.** Frank R. Elevitch and Joel G. Brunson,\* University of Minnesota Medical School, Minneapolis, Minn.

It has been suggested that staining of smears by the fluorescent dye acridine orange (AO), in combination with conventional Papanicolaou criteria for recognition of malignant cells, might reduce the necessity for highly trained cytoscreeners as well as the time required to examine smears. Staining is rapid and simple. Malignant cells are identified easily by a characteristic intense red-orange fluorescence, while other cells fluoresce green to orange.

Vaginal aspirations and cervical scrapings from 641 patients (864 smears) were stained by this method and screened by an observer untrained in cytology. The slides were classified as positive or negative without knowledge of clinical, biopsy or Papanicolaou diagnoses. Ten slides could be stained and read in one hour. Ninety-eight per cent (603 in 614) of the cases with benign lesions were correctly eliminated. There was a 58 per cent (7 in 12) accuracy (correct AO positives among known positives). Of 18 patients classified as AO positive,



7 (44 per cent) actually had malignant lesions. Most AO false positives (6 in 11) occurred in patients with post-irradiated squamous cell carcinoma of the cervix. Reasons for false negatives are discussed.

This technique appears to be valuable in screening a normal population, in that there is a low percentage of false positive results. It does not appear to be of value in screening post-irradiated patients for recurrence. In this study 70 per cent (7 in 10) of primary malignant lesions were identified properly by AO fluorescence of cytoplasm.

AN AUTORADIOGRAPHIC STUDY OF A HUMAN BRAIN AND GLIOBLASTOMA MULTIFORME FOLLOWING THE *in vivo* UPTAKE OF TRITIATED THYMIDINE.† H. A. Johnson, W. E. Haymaker,\* J. R. Rubini, T. M. Flidner, S. W. Lippincott,\* V. P. Bond, E. P. Cronkite, and W. L. Hughes, Brookhaven National Laboratory, Upton, N.Y., and the Armed Forces Institute of Pathology, Washington, D.C.

Thymidine is a specific precursor of deoxyribonucleic acid (DNA). After incorporation into DNA, thymine and its tritium tag do not exchange. The label is diluted only by subsequent cell division. The low energy beta emission (0.018 mev) produces high resolution autoradiographs. Labeling occurs only during DNA replication.

A comatose male patient received 8 injections of tritiated thymidine distributed over a 6-month period. The last dose was given 4 hours before death. At necropsy, a massive glioblastoma multiforme was found. Autoradiographs of tissue showed nuclear labeling in some cells of all sections examined. Since chromosomal replication is normally followed by cell division, the percentage of labeling of a given cell type was an index of the proliferative activity of that cell type. Numerous neoplastic cells were labeled, particularly various sized mononuclear cells, apparently of neuroglial origin. Labeling of hyperchromatic and giant cells may indicate production of polyploidy rather than true cell duplication. Active DNA synthesis was also demonstrated in non-neoplastic astrocytes and perhaps in other glia, in vascular endothelium, in choroid plexus epithelium, and in Schwann cells of cranial nerves.

STUDIES ON THE ANEMIA OF TUMOR-BEARING HAMSTERS. Joseph D. Sherman and Gilbert H. Friedell, Massachusetts Memorial Hospitals, Boston, Mass.

Previous studies have shown that the growth of a transplantable sarcoma in the golden hamster was associated with the development of anemia of moderate degree after 25 days of growth. This occurred although there was neither cachexia nor obvious infection, nor any evidence of hemorrhage into the tumor or elsewhere. It was noted, however, that the onset of the anemia followed the appearance of necrosis within the tumor. The cause of this anemia has been investigated by *in vivo* and *in vitro* experiments.

One group of 45 non-tumor bearing hamsters received repeated injections of a sterile, cell-free extract of necrotic tumor, while another group of 50 animals received injections of a sterile, cell-free extract of viable tumor tissue over a similar period of time. The animals receiving extracts of the necrotic neoplasm developed an anemia quite similar in type and degree to that produced by growing tumor, whereas the animals receiving extracts of viable tumor showed only a slight drop in the average hemoglobin level.

*In vitro* studies were performed to determine the presence or absence of a hemolysin within the tumor, and, if present, its localization in the necrotic or viable portions of the tumor. Sterile, cell-free extracts were prepared from portions of whole tumor, portions of necrotic tumor tissue, and portions of

† Supported by the United States Atomic Energy Commission.

viable tumor. The extracts were then incubated with washed red cells obtained from normal hamsters and from hamsters bearing various homologous and heterologous tumors. A definite lytic effect against all of the test red cells was found in the extracts of viable tumor tissue, with lesser degrees of hemolysis produced by extracts of whole tumor tissue. Extracts of necrotic neoplastic tissue had no direct hemolytic effect.

**LIFE SPAN OF LIVER CELLS: AUTORADIOGRAPHIC STUDIES IN NORMAL AND FATTY RAT LIVER.** Richard A. MacDonald and G. Kenneth Mallory,\* Mallory Institute of Pathology and Harvard and Boston University Schools of Medicine, Boston, Mass.

Information concerning the duration of survival of liver cells in the intact animal has not previously been available. In this study, tritiated thymidine, which is incorporated into deoxyribonucleic acid (DNA), was injected into rats as a means of labeling cell nuclei being formed at the time of the injection. Since DNA is constant until the cell divides further, or dies, and since the half-life of tritium is 12.5 years, the tritium label persists throughout the life of the cell. By determining the rate of disappearance of labeled cells, the average life span of liver cells was calculated. To quantitate and determine changes in the number of labeled cells, a very small biopsy of the liver, and hepatic tissue obtained when the animal was sacrificed at a later time were compared in autoradiographs.

In terms of survival, two populations of hepatic cells were present. The first group had a life span of  $28.2 \pm 25.5$  days in the normal liver, and  $27.7 \pm 14.3$  days in the fatty liver. The second group, which appeared to be those cells that had survived beyond the initial period, had a life span of at least several months.

**IRON ABSORPTION IN RATS FED A PROTEIN-FREE DIET.**† Janis V. Klavins, Thomas D. Kinney\* and Nathan Kaufman,\* Western Reserve University School of Medicine and Cleveland Metropolitan General Hospital, Cleveland, Ohio.

Young male albino rats were fed synthetic protein-free diets supplemented with 2 per cent iron citrate for 4 weeks. Control animals were pair-fed with diets containing 18 per cent casein. Animals on protein-free diets supplemented with iron absorbed significantly less iron than pair-fed controls given a diet containing 18 per cent casein. In addition, the hemoglobin levels in animals fed the protein-free diets were markedly lower than those of the control animals. The results suggest a relationship between dietary protein levels and iron absorption.

**THE NATURE OF HEMOSIDERIN GRANULES IN IDIOPATHIC HEMOCHROMATOSIS: ELECTRON MICROSCOPY, CHEMICAL AND SEROLOGIC STUDIES.** Goetz W. Richter,\* The New York Hospital-Cornell Medical Center, New York, N.Y.

Hemosiderin granules were isolated at necropsy from the liver in two cases of idiopathic hemochromatosis by means of differential centrifugation in sucrose solutions. The granules were identified by light microscopy and chemical tests for iron; the neatness of separation was checked with the electron microscope. They contained 10 to 30 per cent protein, and up to 45 per cent iron; they were almost insoluble in water and poorly soluble in concentrated alkali. Electron microdiffraction studies indicated the presence of hydration products

† This work was supported by a United States Public Health Service Grant No. A-1388.

of  $\alpha\text{-Fe}_2\text{O}_3$  while electron microscopy showed that much of the iron was in the characteristic form of ferritin iron micelles. The protein component of ferritin, apoferritin, seemed evident in shadowed preparations, as described by Farrant, but precipitin tests with serums against apoferritin (tube dilution and agar-gel methods) demonstrated only traces of apoferritin in aqueous extracts of the hemosiderin granules. By contrast, dilute extracts of crystalline ferritin, prepared from the two livers by the method of Granick contained minute amounts of apoferritin and were more active antigens. Solubility curves, obtained with samples of the crystalline ferritin, suggested that soluble ferritin was extracted from hemosiderin granules during their separation from tissue homogenates.

When taken together with previous evidence on the molecular structure of ferritin, on hydrates of  $\alpha\text{-Fe}_2\text{O}_3$ , and on the fine structure of cells laden with hemosiderin, the observations indicated that in the isolated hemosiderin granules ferritin was present largely in a form which contained degraded apoferritin. The evidence indicated further that the deposition of hemosiderin in idiopathic hemochromatosis is closely related to the intracellular metabolism of ferritin in this condition.

OBSERVATIONS ON LUPOID HEPATITIS AND THE HEPATIC LESIONS OF SYSTEMIC LUPUS ERYTHEMATOSUS. I. R. Mackay, L. I. Taft, and D. C. Cowling, The Walter and Eliza Hall Institute of Medical Research and the Royal Melbourne Hospital, Victoria, Australia.

Fourteen cases of lupoid hepatitis (clinical, biochemical and liver biopsy evidence of chronic hepatitis associated with a positive LE cell test) were studied. They frequently displayed a variety of other manifestations of lupus; namely, a characteristic age and sex incidence, skin rashes (4), arthralgia (7), nephritis (3), colitis (3), pleural effusion (2), hemolytic anemia (2), thrombocytopenic purpura (1), lymphadenopathy (1), myocarditis (1) and complement fixing antibodies to human tissue antigens (9 out of 10 cases tested). Hepatitis was the initial and dominant lesion, progressive in its course, despite evidence of suppression of hepatocellular necrosis by cortisone in some patients. The manifestations of lupus were generally mild, transitory and limited in number, but in two patients were so pronounced as to suggest the diagnosis of systemic lupus erythematosus.

By contrast, among 19 patients with systemic LE in whom the liver was examined histologically, only two were found to have chronic hepatitis. In the remainder, the liver was normal or showed a variety of mild and insignificant lesions.

The hyperglobulinemia, positive LE cell test, dense lymphocyte and plasma cell infiltrations in the lesions, complement fixing antibodies to human disease antigens, the relapsing and progressive course frequently suppressed by cortisone, and the overlap in manifestations in lupoid hepatitis and systemic LE indicate similarities in pathogenesis, possibly an abnormal immunologic responsiveness with autodestruction of host tissue.

At present, direct laboratory evidence of such an immunologic anomaly is scanty. It is uncertain if this process, which we have termed "autoclasia," can be extrapolated to other cases of chronic hepatitis, or initiated by liver injury due to virus or malnutrition.

GALL BLADDER AND BILE DUCT ADENOCARCINOMAS IN DOGS, FOLLOWING CHRONIC FEEDING OF ARAMITE.® Stephen S. Sternberg,\* Hans Popper,\* Bernard L. Oser and Mona Oser, Memorial Center, Mt. Sinai Hospital, and Food and Drug Research Laboratories, New York, N.Y.

Few attempts to produce bile passage carcinomas in dogs have heretofore been successful. In the current experiments, these lesions were regularly produced in dogs receiving high doses of Aramite® (B-p-tertiary butyl phenoxy-a-methyl B'-chlorodiethyl sulfite), a miticide. In experiments to establish the safety of residues on certain food crops of Aramite levels of 1 p.p.m., 24 dogs were fed amounts ranging from 500 to 1,500 p.p.m. in otherwise adequate diets; 12 dogs on basal diet served as controls. In the 46 months since the inception of the study, 17 of the treated dogs either died or were sacrificed *in extremis* (462 to 1,220 days). Loss of appetite, progressive weight loss, jaundice and ascites were observed. In the biliary tract (intrahepatic bile ducts, gall bladder, cystic and common ducts, and the ampulla of Vater) of 13 dogs, one or more well differentiated adenocarcinomas were found. Some were papillary, and others mucus producing. Lesions associated with the neoplasms were atypical (pre-cancerous) alterations in both the intra- and extra-hepatic bile ducts and mucosal hyperplasia and adenomas in the gall bladder and its ducts. Carcinoma *in situ* was also encountered in these structures. Although the carcinomas were frequently locally invasive, only one metastasis (in a kidney) was detected. All dogs showed hepatic damage of varying severity, characterized by degeneration and necrosis as well as localized and diffuse regeneration of hepatic cells and ductal and ductular proliferations. In 5 animals the lesions resembled those induced by ethionine. Nine animals exhibited hyperplastic hepatocellular nodules of varying size; some were slightly atypical cytologically, but none were malignant. Only one dog had true cirrhosis. When tumors had obstructed the duct system, bile stasis was prominent; bile lakes and infarcts were noted.

Administration of similar large amounts of Aramite® to rats (which lack a gall bladder and sphincter of Oddi) resulted in hyperplastic and neoplastic nodules in the liver without involvement of the biliary tract. This suggests that the biliary passage carcinomas in dogs result from concentration of carcinogenic material or its metabolic products in the bile.

**GRANULAR CELL NODULES OF THE GASTROINTESTINAL TRACT.** Jacob Churg\* and John Work,\* Barnert Memorial Hospital, Patterson, N.J., and Mountainside Hospital, Montclair, N.J.

Granular cell nodules ("granular cell myoblastoma," "granular cell neuroma") occur in various parts of the gastrointestinal tract. Two such cases were observed, one showing multiple nodules in the appendix, and the other, a single pedunculated submucosal nodule in the cecum.

The appendiceal nodules were located in the inner muscular coat close to the submucosa. It could be ascertained that the granular cells were modified smooth muscle cells. Electron microscopy revealed that the granules were crescent-shaped, oval or rounded structures 0.5 to 1.0  $\mu$  in greatest diameter. They stained intensely with the periodic acid-Schiff (PAS) reagent and periodic acid-silver methenamine (PA-SM). They also stained with oil red O in both frozen and paraffin sections. In addition, some cells contained large round bodies about the size of red blood cells. These also stained with PAS and PA-SM, and stained with oil red O in frozen, but not in paraffin sections. In confirmation of the work of Hausman, it was established that microscopic nodules of similar nature occurred in over 5 per cent of all appendices.

The cells of the cecal nodule also contained two types of granules, fine and coarse. Their staining reactions were similar to those encountered in granular cell myoblastoma elsewhere in the body. Under the electron microscope, the fine granules consisted of irregular, poorly defined aggregates of minute particles

100 to 200 Å in diameter. The coarse granules were sharply defined, homogeneous, round or oval bodies 0.1 to 5  $\mu$  in diameter.

It is suggested that some of the granular cell nodules of the gastrointestinal tract arise from smooth muscle, and that tumors reported as granular cell myoblastoma may not all have identical histogenesis.

**EFFECT OF GUT SHIELDING ON MORTALITY FROM INTESTINAL RADIATION.** Chandler Smith\* and Simon Koletsky,\* Western Reserve University School of Medicine, Cleveland, Ohio.

The cause of death following heavy doses of radiation to the intestinal tract has not yet been adequately explained. Neither has it been clarified as to just how protection (lead shielding) of a small segment of intestine lowers the mortality from intestinal radiation.

Experiments were undertaken to clarify some of the factors involved in both mortality and protection. By a surgical technique in rats, a segment of ileum with intact blood supply was completely isolated from the rest of the large intestine, and continuity of the intestinal tract was restored by end-to-end anastomosis.

Lethal doses of x-radiation were then given to the abdomens of these animals with and without shielding of the isolated segment of ileum and also with and without shielding of a segment of the intact ileum. A comparison was made of morbidity and mortality in each experimental group. The results indicated that in order for segmental shielding of the intestine to be beneficial, the shielded segment must be in continuity with the rest of the large intestine and not isolated. Elaboration of specific protective substances by the shielded segment appeared to be less important.

**OBSERVATIONS ON THE REGENERATIVE CAPACITY OF INJURED AND TRANSPLANTED STRIATED VOLUNTARY MUSCLES.** Amara Kitiyakara and D. Murray Angevine,\* University of Wisconsin Medical School, Madison, Wis.

The *vastus medialis* muscle of rats was injured by crushing or cutting and examined for evidence of regeneration at intervals of from 24 hours up to 2 months. An injury in which only the contractile structures were destroyed, leaving sarcolemmal nuclei and endomysium tubes intact resulted in maximal regeneration. Severe crushing or cutting usually gave rise to connective tissue replacement due to destruction of structures essential for regeneration.

Small segments of rat muscle were transplanted to either omentum or mesentery in the same animal. There was degeneration of the muscle fibers of such isolated fragments within 24 hours. This was followed promptly by regeneration, indicating that the growth of adjacent muscle or the presence of muscle stumps was not essential to the process.

Ribonucleic acid (RNA) content and the uptake of  $P^{32}$  were studied by cytochemical and autoradiographic procedures. It was found that regenerating fibers resembled embryonic tissues or protein synthesizing cells in RNA metabolism.

**IMMERSION PROTECTION AGAINST 15 MINUTES OF 10,000 G IN HYPOTHERMIC SUSPENDED ANIMATION.** B. Black-Schaffer\* and G. Hensley, University of Cincinnati College of Medicine and Cincinnati General Hospital, Cincinnati, Ohio.

When baby mice (8 to 12 days old), in hypothermic suspended animation, were centrifuged, the mechanical effects of acceleration upon the tissues were readily separated from the effects upon function, such as respiration, cardiovascular dynamics, and metabolism. Under these circumstances, the highly

effective protection against acceleration by immersion to the limits of our apparatus, approximately 10,000 G, was readily demonstrated. It was shown that the baby mice thus protected could survive a stress which, when translated into a hypothetical rectilinear velocity, would at the end of 15 minutes impart a constant speed of about 23,500 kilometers (14,592 miles) per second. At such a velocity a relativistic time dilation of 1 per cent would occur.

#### READ BY TITLE

**THE EFFECT OF GROWTH HORMONE AND OF PARTIAL HEPATECTOMY ON EXPERIMENTAL DIETETIC LIVER NECROSIS.** Kurt Aterman, Dalhousie University, Halifax, N.S., Canada.

Weanling rats fed a diet containing yeast as the sole source of protein, died of hepatic necrosis. This course could be significantly influenced by various nutritional (vitamin E, selenium, cystine, cod liver oil) and hormonal (cortisone, ACTH, aldosterone, thyroid powder) factors. In the present experiments the effect of growth hormone was studied. Given in adequate amounts, two different preparations of growth hormone in each case produced a significantly earlier onset of fatal liver necrosis in the treated rats ( $17.2 \pm 0.7$  days) than in their control litter mates ( $28.4 \pm 4.2$  days) without affecting the rate of growth or food intake. This effect was accompanied by marked, presumably fatty, vacuolation of the liver cells, and occasionally also by a pronounced, but localized hemorrhage into the wall of the large intestine. The accelerating effect of growth hormone resembled that of thyroid hormone, the only other hormone found to shorten the survival time ( $30.0 \pm 2.6$  days as compared to  $17.3 \pm 1.4$  days). In view of the known contamination of pituitary growth hormone preparations with thyrotrophic hormone, the thyroid glands of the treated rats were examined in each case, but no histologic evidence of stimulation could be found. Possible modes of action of growth hormone are discussed.

Partial hepatectomy also accelerated the onset of fatal liver necrosis, presumably because of the increased requirements of protective factors during the period of hepatic restoration. It is possible, however, that the operative removal of a large part of the hepatic stores of the protective substance(s) may have been the deciding factor in this experiment.

**POSSIBLE EFFECTS OF ALTITUDE ON THE OCCURRENCE OF CHRONIC THROMBOSIS OF MAJOR PULMONARY ARTERIES.** Morgan Berthrong\* and Autrey R. Croke, Glockner-Penrose Hospital, Colorado Springs, Colo.

Chronic thrombosis of the major pulmonary arteries is a relatively rare condition which has been reported with an incidence of approximately 1 to 2 cases per 1,000 necropsies. In this hospital over the past 10 years, however, 15 instances of this condition have been found among 1,000 necropsies. The 15 instances occurred among 35 patients who died with chronic cor pulmonale, the result of abnormality of the lungs or its vessels and was unrelated to other forms of heart disease. Four examples were seen in patients with chronic tuberculosis, where such thrombi are a recognized complication. At least 5 were found in patients with pulmonary emphysema. In this condition, thrombosis of the major pulmonary arteries is not usually considered to be common but was found in our hospital in 5 of the 13 patients with pulmonary emphysema and chronic cor pulmonale. Pulmonary artery thrombosis was present in 3 patients as a consequence of emboli from phlebothrombosis; all 3 died of cor pulmonale. Three other cases were found among patients dying of cor pulmonale, 2 with pneumoconiosis and 1 with "primary" pulmonary hypertension. The relative frequency of chronic thrombosis of the major pulmonary arteries in patients in this community may be related to the altitude. While conditions at



6,000 feet have not been thought to place a burden on normal individuals, patients with chronic lung disorders may do poorly at this altitude. Polycythemia has occurred in patients with greater frequency in Colorado Springs than at lower altitudes as indicated by its presence in 9 of 13 patients with pulmonary emphysema and cor pulmonale. Polycythemia apparently did not play a role in the cases of tuberculosis or primary pulmonary vascular disease but was present in both patients with pneumoconiosis. The clinical and pathologic aspects of chronic pulmonary artery thrombosis in patients dying of cor pulmonale are presented.

**VERNIX GRANULOMAS OF THE AMNION IN OLIGOHYDRAMNIOS; MORPHOGENESIS AND CLINICAL IMPLICATIONS.** William A. Blanc,\* Columbia-Presbyterian Medical Center, New York, N.Y.

Amniotic nodules (*Amnionknötchen*, *amnion nodosum*) appear as innumerable round, raised, yellowish plaques studding the amniotic surface. They vary from lenticular size down to barely visible and easily missed dots. Sixteen instances have been published since 1889. The data gained from the study of 10 complete examples of this condition, collected within 4 years, provide further information on the incidence, pathogenesis and clinical significance of this supposedly rare lesion.

Clumps of vernix were deposited on the amniotic surface, either by direct contact with the fetal skin or as a consequence of the high concentration of desquamated vernix suspended in an abnormally small amount of amniotic fluid. The underlying amniotic epithelium underwent necrosis. The mass of vernix was then invaded by fibroblasts and histiocytes from the amniotic mesoderm. The nodule exhibited some hyalinization and was fixed further to the placenta by the growth of amniotic epithelium over its edges.

Oligohydramnios was actually observed or highly probable in 8 cases. Six of these had severe genito-urinary anomalies, ranging from urethral obstruction to renal aplasia and polycystic kidneys. The other two placentas had severe circummargination. In the remaining two cases, there was prolonged retention of a dead fetus after rupture of the membranes.

The recognition of vernix granulomatosis, apparently a good evidence of oligohydramnios, might be of help in the investigation of the latter condition. Vernix granulomas may also provide clues to the clinician, stimulating the search for anomalies.

**GLOMERULOSCLEROSIS ASSOCIATED WITH CARDIAC VALVULAR DISEASE.** J. M. B. Bloodworth, Jr.,\* Ohio State University, Columbus, Ohio.

Because of the probable role of the endothelial cell in degenerative vascular disease and the unusual concentration of this cell in the renal glomerulus, we have undertaken a detailed investigation of degenerative glomerular alterations. The term "diffuse glomerulosclerosis," although a misnomer, has been used to describe a diffuse thickening of the glomerular stroma. The nodular thickening of diabetic glomerulosclerosis and the hilar thickening of arteriolar sclerosis were not included. This lesion was not uncommon, occurring in about 10 to 20 per cent of all kidneys examined at necropsy. Bell reported diffuse glomerulosclerosis in 50 to 60 per cent of all diabetic patients, and recently it has been reported in 70 to 80 per cent of patients with advanced cirrhosis of the liver. We have found an increased incidence in patients with aortic insufficiency. Diffuse glomerulosclerosis was present in approximately 90 per cent of the patients with syphilitic aortic insufficiency and in 55 per cent of patients with rheumatic aortic insufficiency. Diffuse glomerulosclerosis was also present in approximately 75 per cent of patients with syphilis without cardio-



vascular involvement. In cases of rheumatic valvular disease without aortic insufficiency, the incidence of diffuse glomerulosclerosis was only 33 per cent.

The lesion began as a proliferation and anaplasia of the endothelial cells which then became enlarged and eosinophilic, partially obstructing the glomerular capillaries. At the same time the stroma contained an increased amount of PAS-positive fibrillar material which gave most of the stains for collagen but was morphologically different. Finally the endothelial cells merged with the thickened stroma, which contained many nuclei without cell borders. Some lipid was present. Clinical correlations and implications are discussed.

**ABORTIVE FORMATION OF ENAMEL IN AMELOBLASTOMA.** P. E. Boyle\* and V. Kalnins, Western Reserve University School of Dentistry, Cleveland, Ohio.

Abortive enamel formation has been observed in constantly erupting teeth of rodents. This occurs when ameloblasts become detached from growing enamel matrix because of some pathologic conditions. Dislocated into periodontium, ameloblasts continue to produce enamel matrix in the form of single or multiple drops (or pearls) of various sizes. Some of these drops become calcified. Development of enamel has been recognized hitherto in odontomas but not in ameloblastomas. It has been believed that there is no formation of enamel in ameloblastoma since the dentin is not formed in this neoplasm. Our observations showed that enamel could develop in the absence of dentin in well advanced ameloblastomas. Ameloblastoma cells produced enamel matrix as drops of various sizes. Formation of young and transitional enamel matrix was observed. Mineralization of these drops was not found.

**HEPATIC FIBROPLASIA IN NORMAL AND SCORBUTIC GUINEA PIGS.** Nijole-Virginija Brazenas, Fiorenzo Paronetto, Arthur W. Ludwig and Frederick G. Zak,\* The Mount Sinai Hospital, New York, N.Y.

Previously the influence of carrageenin (a sulfated polygalactose) on focal hepatic fibrosis was studied in normal rats and in those given cortisone or ethionine. Because of the importance of ascorbic acid in fibrogenesis, these studies were extended to scorbutic guinea pigs into which 0.5 ml. of a 0.3 per cent solution of carrageenin was injected beneath the liver capsule. The guinea pigs were sacrificed 2 to 12 days later, and the livers were fixed in formol-alcohol.

Non-scorbutic animals showed extensive necrosis early, with neutrophil reaction. Intensive proliferation of ductular cells and fibroblasts began on the fourth day. Simultaneously, much PAS-positive (but diastase resistant), Alcian blue-positive ground substance and numerous argyrophilic fibrils appeared. Later the lesions diminished in size with partial fibrous replacement.

In scorbutic animals coagulation necrosis was more severe throughout the experiment with a persisting sharp demarcation between necrotic and viable liver substance. Fibroblast and ductular cell proliferation was scant at all stages. However, abundant, intensely PAS-positive and Alcian blue-negative material, possibly from serum, was laid down at the periphery of the necrotic zones and underwent partial calcification although the level of serum calcium was unchanged. Argyrophilic fibers were scant and thin. In ascorbic acid deficiency, hepatic fiber formation in the presence of fibroblasts was similar to that encountered in the skin.

**TRYPAN BLUE-INDUCED TUMORS OF RATS: MORPHOLOGIC, HEMATOLOGIC, AND SEROLOGIC OBSERVATIONS.** David V. Brown and Lorna M. Norlind, Veterans Administration Hospital, Seattle, Wash.

Prolonged injection of a dilute solution of trypan blue produced neoplasms

of the reticuloendothelial system in the rat. These tumors appeared as histiocytomas of the liver and reticulum cell sarcomas of the lymph nodes, bone marrow, and spleen. Animals subjected to whole-body irradiation prior to the administration of dye were found to develop fewer tumors.

Regular periodic hematologic studies were carried out to follow the profound alterations which occurred in the blood of the tumor-bearing animals. Serum proteins were determined at appropriate intervals and correlated with alterations in the animal's ability to produce antibodies.

The present report is concerned with an evaluation of the histogenesis and nature of these experimentally induced reticuloendothelial tumors and with the correlations which could be made with the hematologic and serologic data.

**RENAL HOMOGRAFT REJECTION PATTERNS IN MAN AND THE EXPERIMENTAL ANIMAL.** Gustave J. Dammin,\* Joseph E. Murray and John P. Merrill, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Mass.

In the canine renal homograft, during its functional survival, there was a regular and characteristic sequence of morphologic alterations. Mononuclear cell infiltration of the interstitial tissue occurred while function was good, and became more marked, particularly in a perivascular distribution, as function failed. This usually occurred by the seventh day. The glomeruli remained essentially normal. There was progressive atrophy of the tubules and occasionally an arteritis developed. This was characterized by subendothelial and mural infiltration by pyroninophilic mononuclear cells which resembled those seen in the perivascular infiltrate.

In the patient with chronic uremia, the course was more variable, and functional survival of the renal homograft might continue for several weeks to months. The pattern resembled that described above but was focal and developed gradually, with arteritis a relatively more prominent component. Lumen narrowing accompanied marked intimal thickening. There was disruption of elastica but no fibrinoid deposition.

To a patient who had had the only renal tissue removed, 600 r. of total body radiation were administered. This was followed by the intravenous administration of homologous marrow and the transplantation of a renal homograft. A moderate level of function was achieved by the renal homograft, and biopsy specimens of the homograft on the third and 25th days after transplantation did not show a rejection pattern. When the patient succumbed on the 28th post-transplant day, there was still no rejection pattern, indicating a prolonged functional survival and also suggesting that the cells which infiltrated the renal homograft originated in the recipient rather than in the homograft itself.

**THE ROLE OF THE THYMUS IN THE DEVELOPMENT OF MURINE LEUKEMIA FOLLOWING RADIATION.**† James T. Duhig, New England Deaconess Hospital, Boston, Mass.

The importance of the thymus in the development of murine leukemia was indicated by the demonstration of thymic production of a lymphocytosis-stimulating factor and by the development of leukemia in a normal thymus transplanted into an irradiated thymectomized host. The influence of the thymus on the development of leukemia was studied in 1,000 A strain mice divided into 4 groups. The first group served as controls; the remaining 3 groups received doses of x-irradiation of 100 r. weekly to totals of 500 r., 1,000 r., and 1,500 r. respectively. At their natural death, necropsy was performed on

† This work was done under the United States Atomic Energy Commission Contract AT(30-1)-901 with the New England Deaconess Hospital.

the mice. Increasing doses of radiation appeared to decrease the life span of the mice, one of the causes being lymphoid leukemia. In mice suffering from this disease, depending on whether or not the thymus was enlarged, two major groups were distinguished: thymic leukemia and non-thymic leukemia.

The incidence of thymic leukemia was very low (1 per cent) in control mice but was greatly increased by irradiation and by increasing the dosage. The severity of the disease, judged by infiltration of leukemic cells into other organs and tissues, was also increased by irradiation. The incidence of non-thymic leukemia was little affected by irradiation, and at higher doses the incidence even appeared depressed below the control level of 11 per cent. Radiation appeared to have no influence on the severity of the disease. These observations emphasize the important role of the thymus in the development of murine leukemia and indicate that the pathogenesis of the two diseases described may be quite different despite similar microscopic appearances.

**ELECTRON MICROSCOPY OF EXPERIMENTAL PITUITARY TUMORS.**† Marilyn G. Farquhar and Jacob Furth,\* University of California School of Medicine, San Francisco, Calif., and the Children's Cancer Research Foundation, Boston, Mass.

Experimental thyrotrophic (TtT), adrenotrophic (AtT), and mammosomatotropic (MtT) pituitary tumors were examined by electron microscopy to ascertain what cytologic features distinguished these lesions and to compare the structure of dependent and autonomous variants composed of the same cell type.

Cells from highly dependent TtT appeared similar to "thyroidectomy cells" of the rat or mouse. Like "thyroidectomy cells," TtT cells were large, and their cytoplasm was filled with numerous vesicles of variable size. There was a prominent Golgi zone but only a few secretory granules. In autonomous strains, vesicles were not so numerous or closely packed. The observations suggested the derivation of TtT from "thyroidectomy cells" as proposed by Halmi and Gude.

Cells from AtT were characterized by small size, relative absence of cytoplasmic vesicles, and small to moderate amounts of cytoplasm containing relatively few formed elements (mitochondria, Golgi elements and endoplasmic reticulum). AtT cells resembled those from the normal rat or mouse intermediate lobe of the pituitary more closely than any anterior lobe cell.

The most distinctive features of MtT cells were their dense, ovoid or globular cytoplasmic granules. Some granules were identical in size (350 to 600  $m\mu$ ) and appearance to acidophil granules in the normal rat or mouse pituitary. Others were much larger (several microns). MtT cells showed relatively pale mitochondria and abundant endoplasmic reticulum, varying from tiny ovoid profiles to the parallel arrays of cisternae characteristic of normal acidophils. From these observations, it seemed likely that MtT were derived from acidophils.

In general, greater autonomy (and loss of functional activity) was associated with progressive loss of identifying features. There was also great variability in cell size and in the form of cellular components; complicated nuclear infoldings and aberrant mitochondrial forms with randomly arranged internal crests were frequently seen.

**THE EFFECT OF CORTISONE AND ADRENALECTOMY ON HEPATIC METASTASES FOLLOWING THE INTRAPORTAL INJECTION OF WALKER 256 CARCINOSARCOMA.** Edwin R. Fisher\* and Bernard Fisher, University of Pittsburgh, Pittsburgh, Pa.

† Supported by Grants P-112 and 113 from the American Cancer Society and by Grant C-2259 from the National Cancer Institute.

Although there is general agreement that cortisone exerts an inhibitory effect on the local growth of various transplantable tumors in experimental animals, the results of investigations concerned with the effect of this agent on the development of metastases are diverse. In the present investigation, cortisone was administered to rats that received direct intraportal injections of known numbers of Walker 256 carcinosarcoma cells. The incidence of hepatic metastases 2 weeks following injection was significantly reduced in animals receiving 2 mg. of cortisone for 2 weeks prior to and after injection. A similar dose for only 3 days prior to the administration of tumor cells or a larger dose (10 mg.) failed to influence the incidence of metastases. There were no perceptible morphologic or histochemical differences noted in the livers or neoplasms of these groups. The results do not appear related to alterations in liver composition or the general catabolic effect of cortisone as evidenced by the study of appropriate controls. Adrenalectomy performed one week prior to the injection of tumor cells similarly failed to alter the incidence of hepatic metastases or their increased incidence following procedures observed to produce such an effect. Reported increases in metastases from local tumor implants following the administration of cortisone would appear related to the effect of this agent on the primary growth. Moreover, cortisone appeared to possess an optimum dosage relative to its effect on the development of hepatic metastases. Stress did not seem to represent a factor in this phenomenon.

**LUNG CANCER IN CHROMATE WORKERS.** Russell S. Fisher\* and Peter W. Rieckert, Office of the Chief Medical Examiner, Baltimore, Md.

A series of 40 bronchogenic carcinomas in 38 chromate workers studied between 1938 and 1958 is reported. All patients were males and were employed for a minimum of 5 to a maximum of 49 years in various divisions of a plant producing refined chromates from ore. Thirty-one showed perforation of the nasal septum, 6 did not, and in 1 this information was not available. In 6 patients, there was no history of smoking; 2 did not smoke; 3 were pipe or cigar smokers; 3 smoked less than one pack of cigarettes per day; 19, from one to one and a half packs, and 5, in excess of 2 packs daily. Eighteen had had definitive surgical treatment; 3 of the 7 survivors have now lived more than 6 post-operative years. The sites of the primary tumors were: Left lung, 13 (upper lobe, 5; main bronchus, 4; lower lobe, 4). Right lung, 25 (upper lobe, 11; middle lobe, 2; main bronchus, 6; lower lobe, 6). Two patients had a second cancer; one of these had a primary neoplasm in the left main bronchus and a second tumor in the right upper lobe. The first was a squamous cell carcinoma; the second was oat cell in type. The other patient had a squamous cancer in the right main bronchus and 4 years later another in the left lower lobe bronchus. Thus, of the 40 cancers, 14 were in the left lung and 26 in the right. Histologically, the tumors were classified as squamous cell type, 26; oat cell type, 8; and undifferentiated type, 6.

**EFFECT OF 4-AMINO-PTEROYLGLUTAMIC ACID (AMINOPTERIN) ON RAPID PHYSIOLOGIC CELL GROWTH (RAT PLACENTA).**† Alvan G. Foraker,\* Robert N. Wilcox and Genevieve Marino, Baptist Memorial Hospital, Jacksonville, Fla.

Rapidly growing cells in the mammalian placenta offer a discrete, inexpensive test site for studies in cell injury by methods employed in tumor therapy. In furtherance of this approach, 4-amino-pteroylglutamic acid (10 mg. per kg.) was administered intraperitoneally to 18 Wistar rats on the ninth day of a 21-

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day gestation period. On the 13th day, the right uterine horn was removed. The rats were sacrificed at varying intervals subsequently, for study of the left uterine horn. Seven pregnant, untreated rats and 9 non-pregnant, treated rats were controls. Tissues from the placenta, uterus and ovary were stained by the hematoxylin and eosin, periodic acid-Schiff (undigested and amylase-digested), Snook's reticulin, Verhoeff's elastic, and Masson's trichrome techniques and also processed to demonstrate acid and alkaline phosphatase (azo dye method). Death and disintegration of embryos was induced by 4-amino-pteroylglutamic acid almost uniformly. Fetal elements of the placenta underwent varying degrees of necrosis. Adjacent maternal elements of the placenta showed some degeneration, with fragmentation of reticulin fibers. PAS-positive, amylase-resistant material (apparently fluid) accumulated in the uterine cavity around the degenerating tissue. In ensuing days, necrosis and disintegration of all placental elements proceeded, with residual inflammation and hemofuscin deposition in the uterine wall. Utilization of mammalian placenta for studies in cell injury, especially by chemotherapeutic agents, is discussed.

EFFECTS OF HYPOPHYSECTOMY, ADRENALECTOMY, AND SUBSTITUTION THERAPY IN GOLDEN HAMSTERS WITH AN OBLIGATE INTRACELLULAR INFECTION (*Besnoitia jellisoni*).† J. K. Frenkel,\* University of Kansas Medical School, Kansas City, Kan.

The frequent occurrence of adrenal infection, simulating the tuberculous and histoplasmic adrenal involvement which leads to Addison's disease in man, has made *Besnoitia* infection of hamsters an interesting model. After subcutaneous inoculation, greatest microbial numbers (computed for 20 mg. tissue) occurred in the second week in adrenals (80,000), spleen (13,300), ovaries (4,000), liver (550), and brain (350), and in the third week in eyes (10,000) and lungs (5,500). Infection persisted in the chronic state, with most organisms found in adrenals (often with gross necrosis), brain, eyes, and lungs; intermediate numbers were found in liver, testes, and kidneys; lowest numbers appeared in spleen, heart, and skeletal muscle. The latter 3 organs and the eyes were frequently free of infection. After hypophysectomy, initial proliferation of *Besnoitia* was decreased in all organs, with the lowest titers in the adrenals and ovaries. Foci of macroscopic adrenal necrosis no longer occurred. Porcine ACTH-zinc (0.5, 1, and 2 units) which promoted adrenal weight, increased the proliferative rate of *Besnoitia* in the adrenal, so that necrosis recurred, although irregularly and to a lesser degree. Foci of adrenal necrosis in intact controls were decreased by ACTH-zinc, whether given alone or with corticosterone to inhibit endogenous ACTH. It is concluded that porcine ACTH is less potent than the endogenous product and that it might change the nature of the adrenal secretory product. Adrenalectomized hamsters, maintained on deoxycorticosterone trimethylacetate (which in itself does not appear to influence infection) lived longer than intact hamsters. When supplemented with cortisol, a daily dose of 60 µg. decreased survival time to that of intact hamsters and decreased thymic weight to near normal.

HYPERSPLENISM AND ANEMIA IN THE TUMOR-BEARING HAMSTER. Gilbert H. Friedell and Joseph D. Sherman, Massachusetts Memorial Hospitals, Boston, Mass.

The growth of a methylcholanthrene-induced transplantable sarcoma in the cheek pouch or the flank of the golden hamster was shown to produce a moderate

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degree of anemia, although there was no cachexia, obvious infection, or grossly discernible hemorrhage. A similar type and degree of anemia was produced in non-tumor bearing animals by repeated injections of a sterile, cell-free extract of necrotic tumor tissue during a 35 to 50 day period. When correlated with the hematologic observations, the results of gross and microscopic examination of the spleen and liver in some 200 experimental animals suggested that the anemia was secondary to hypersplenism induced by products found in necrotic tumor tissue.

Splenomegaly was proportional to the degree of anemia. The amount of reticuloendothelial hyperplasia and anaplasia, the extent of plasma cell production, the amount of hemosiderin present, and the amount of extramedullary hematopoiesis in the spleen were also proportional to the degree of anemia. Other histologic features in the spleens of these animals were also assessed, and their relationship to the development of this anemia of cancer-bearing animals is discussed.

Extramedullary hematopoiesis was less common in sections of liver. When it was present, it was invariably associated with marked anemia and with marked reticuloendothelial hyperplasia and anaplasia in the spleen. Plasma cells were infrequently seen, and the liver did not appear to participate in the immunologic response indicated by the proliferation of cellular elements in the spleen.

**CARDIOVASCULAR CHANGES IN HYPOTHYROIDISM; REPORT OF A CASE OF CRETINISM.** S. A. Goldberg,\* The Presbyterian Hospital, Newark, N.J.

In a study of a female cretin and in experimental cretinism in sheep, marked cardiovascular alterations were encountered.

In the cretin as well as in the experimental animals, the cardiac ventricles were dilated and the myocardium softer than usual. Microscopically, the myocardium was not remarkable. In some of the animals thyroidectomized at an older age, the subepicardial fat showed myxedema.

The arch of the aorta and the coronary arteries of the cretin showed atherosclerosis. This was evidenced by hyaline thickening containing cystic foci in the intima of the coronary arteries and cholesterol slits and calcium granules in the aorta. The latter were limited to the arch.

In the experimental animals, the aortas and pulmonary arteries were widened and contained plaques resembling snake skin in appearance. These were formed by a deposition of calcium in the media next to the intima. The intima itself was not affected although the tunica elastica lacked the normal corrugations. Between the calcified plaques there were hyaline thickenings producing the "snake-skin" effect.

It is felt that these alterations were compensatory to the strain exerted on a vascular system with development arrested as a result of thyroidectomy or aplasia of the thyroid.

**THE LOCALIZATION OF RADIOACTIVE SULFUR IN TISSUES OF CHONDROSARCOMA PATIENTS AFTER ADMINISTRATION OF LARGE DOSES.**† Raymond G. Gottschalk,\* Louis K. Alpert, Roy E. Albert and Paul Bell, Jr., George Washington University School of Medicine, Washington, D.C., and Veterans Administration Center, Martinsburg, Va.

It was previously demonstrated that cartilage and cartilaginous tumors had an affinity for radiosulfate ( $S^{35}$ -sulfate) and that large doses of this isotope

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destroyed growing cartilage. In the course of a study of the effects of large amounts of radiosulfate, 3 patients with advanced chondrosarcoma received a total of 555 to 923 mc. in divided intravenous doses. Numerous tissue specimens were obtained by repeated biopsy and at necropsy. The microscopic and radioautographic observations are presented.

Soon after injection, the isotope was selectively fixed in the living portions of the chondrosarcoma and especially at the proliferating edges. The uptake was high and fairly uniform in the myxomatous portions, and it was least in the calcifying and necrotic regions. The fibrous stroma also retained radiosulfur. Several months after injection, significant amounts of  $S^{35}$  were still found in the chondrosarcomas, but the isotope was deposited in some foci inside the necrotic portions and in the stroma. The radioautographic patterns and the time-distribution of the calculated dosage of internal irradiation indicated that a large fraction of the radiation was initially delivered to the proliferating zones. No tumor regression was detected. In view of the variation of structure and viability normally occurring in different parts of chondrosarcomas, it was difficult to ascertain whether the administration of radiosulfur had produced the histologic effects of tumor necrosis comparable to the experimental effects on the epiphyseal cartilage of young animals. Pronounced myxomatous alterations were observed in two patients, but such changes may occur spontaneously.

The radiosulfur injections produced the pattern of hypoplasia followed by regeneration in the bone marrow. The patterns of distribution in numerous other tissues of the body are also demonstrated.

**GEOGRAPHIC PATHOLOGY. CHRONIC MYOCARDITIS IN VENEZUELA.** S. E. Gould,\* Wayne County General Hospital, Eloise, Mich.

A form of chronic myocarditis of adults, clinically and pathologically distinctive, was first recognized in Venezuela about 20 years ago and, later, in a number of other Latin-American countries. Because of its relatively greater frequency in certain parts of Venezuela (in Valencia, up to 43 per cent of deaths among adults, exclusive of deaths from accidents), the disease is of considerable importance. Through the interest and cooperation of pathologists and cardiologists of Venezuela and of the National Heart Institute (Bethesda, Md.), an opportunity was afforded to study at first hand the clinical and pathologic material. This report outlines the principal clinical manifestations of the disease and describes and illustrates in detail the pathologic features which characterize this form of chronic myocarditis.

Grossly the heart was prominently enlarged and globose, with dilatation of all chambers and hypertrophy of both ventricles. The myocardium was flabby and lusterless, and often the seat of fibrosis and mural thrombosis. The coronary arteries were free of abnormality, and the lungs and kidneys showed no evidence of hypertension. Microscopically, the myocarditis, present in any or all chambers, was characterized by diffuse, often severe, lymphocytic and frequently histiocytic infiltration. Chief among the other features was the prominent hyperemia and interstitial edema.

The current views of the etiology, including the role of Chagas' disease, are discussed and a program is outlined which is aimed at recognition of the cause and at prevention of the disease.

**A HISTOCHEMICAL EVALUATION OF HETEROTOPIC OSSIFICATION AND DYSTROPHIC CALCIFICATION IN EXPERIMENTAL INFARCTION OF THE RAT KIDNEY.** John Gruhn\* and Edwin R. Fisher,\* University of Pittsburgh and Veterans Administration Hospital, Pittsburgh, Pa.

Ligation of the vascular pedicle of the rat's kidney was followed by dystrophic calcification within one week and heterotopic ossification in the regions adjacent



to proliferating renal pelvic epithelium within 4 weeks. These observations were explored by histochemical techniques to elucidate the role of proliferating epithelium, alkaline phosphatase and acid mucopolysaccharides in these processes. The results indicated that dystrophic calcification was not dependent upon the presence of alkaline phosphatase but occurred in sites depleted of such enzymic activity. Acid mucopolysaccharide of the chondroitin sulfate type was demonstrable at the same sites in the same temporal sequence as reactions for calcium. This acid mucopolysaccharide could act as an exchange resin to concentrate calcium. Ossification was preceded by marked proliferation of renal pelvic epithelial cells which accumulated abundant ribonucleoprotein and alkaline phosphatase. Subsequently, local fibroblasts were observed to proliferate, to develop intense alkaline phosphatase activity and to become osteoblasts. These, when mature, contained less alkaline phosphatase than precursor cells. In no instance could the acid mucopolysaccharide of bone matrix be related to that observed in renal ground substance. It was concluded that proliferating renal pelvic epithelium and its alkaline phosphatase stimulated local fibroblasts to assume osteoblastic function, thus playing a role in bone matrix formation rather than its calcification. That the phenomena of dystrophic calcification and heterotopic ossification were local in nature was apparent from the normal serum values for alkaline phosphatase and calcium observed throughout the experimental period. The histochemical features of heterotopic ossification in infarction of the renal parenchyma were the same as those of normal membranous bone formation.

SEQUENCE OF ALTERATIONS IN KIDNEYS AND IN ELECTROPHORETIC PATTERNS OF SERUM AND URINE OF NEPHROTIC RATS. Cornelia Hoch-Ligeti,\* University of Virginia School of Medicine, Charlottesville, Va.

This investigation was prompted by the observation that in the urine of nephrotic children with high serum lipoprotein, no lipoprotein could be demonstrated.

Seventy young adult Wistar rats were rendered nephrotic by daily injection of aminonucleoside; 20 untreated rats served as controls. Urine and blood were collected daily prior to sacrificing the rats. The protein and lipoprotein distribution of serum and urine was investigated electrophoretically. The water and fat content, the enzymatic reduction of neotetrazolium, alkaline phosphatase and mucoproteins were determined chemically or histochemically in the kidneys and livers.

The total serum protein in the rats and the enzymatic activities of the kidneys decreased slightly the day after treatment was begun and were markedly reduced after 6 days. Protein was found in the urine from the sixth day onward. After 10 days, ascites was present in all animals. The dry weight of the kidneys decreased from the tenth day onward. The water and fat content and the enzymatic activities of the liver remained unchanged before and after ascites developed.

Stainable lipoproteins occurred in the serum after the sixth day. Simultaneously, fat droplets were observed in the glomerular capillaries; later the tubular epithelium also contained fat. However, on chemical analysis, the fat did not increase in amount. All fractions of serum proteins were excreted in the urine with a preponderance of albumin. As with nephrotic children, no stainable lipoproteins, or only traces of them, were found in the urine.

METABOLIC STUDIES IN ACUTE MYOCARDIAL ISCHEMIC INJURY. R. B. Jennings,\* J. P. Kaltenbach and W. B. Wartman,\* Northwestern University Medical School, Chicago, Ill.

The aerobic metabolism of homogenates of normal and ischemic left ventricle

was compared in 16 mongrel dogs at various time intervals after high ligation of the circumflex branch of the left coronary artery. Eight control dogs were also studied. Glucose and fructose-1,6-diphosphate were used as substrates. Three to 5 dogs were studied at several time periods: 15, 30, 60, and 180 minutes, and 24 hours after ligation. The injured tissue was taken from the posterior papillary muscle (PP) and the non-ischemic tissue from the anterior superior septum (LV). After one hour of incubation with either substrate, there was no difference between the PP or LV in either oxygen consumption or in the levels of organic phosphorus ( $\Delta$  organic phosphorus). Both the oxygen and  $\Delta$  organic phosphorus were slightly decreased in PP at 15 and 30 minutes after ligation. However, between 30 and 60 minutes, there was a sharp decrease in oxygen and  $\Delta$  organic phosphorus to levels 40 to 50 per cent of control values at 60 minutes. A further decrease to less than 15 per cent of normal was observed at 180 minutes, and at 24 hours there was a complete loss of oxidative metabolism in the PP. The break in metabolism between 30 to 60 minutes correlated well with the onset of irreversible injury in the cells of the PP 25 to 30 minutes after ligation. An unexplained observation was a marked increase to 130 per cent of normal in oxygen consumption and  $\Delta$  organic phosphorus in LV 60 and 180 minutes after ligation.

STUDIES ON THE DIRECT AND INDIRECT EFFECTS OF  $C^{14}$ -LABELED BACTERIAL POLYSACCHARIDE ON MURPHY LYMPHOSARCOMA IN THE RAT. Russell S. Jones\* and E. Virgil Howell, University of Utah College of Medicine, Salt Lake City, Utah.

The localization of  $C^{14}$ -labeled bacterial polysaccharide suggested that tumor necrosis might result from the direct effect of such labeled products. To explore the possible indirect roles of the labeled bacterial polysaccharide upon the tumor, 3 groups of experiments were conducted: (a) transfusion into tumor-bearing rats of plasma from animals previously injected with labeled bacterial polysaccharide; (b) injection of tumor extracts; and (c) administration of corticosterone and cortisol.

Criteria of response were lethal effect, tumor necrosis and regression and isotopic uptake from bacterial polysaccharide. The lethal effect of bacterial polysaccharide in rats with large tumors was associated with delayed and decreased binding of the polysaccharide to plasma proteins *in vivo*. The injection of such polysaccharide-containing plasma into tumor-bearing rats induced more necrosis than did similar plasma from normal rats or guinea pigs or a comparable amount of the original polysaccharide in 0.15 M NaCl. *In vitro* the plasma proteins of tumor-bearing rats had decreased capacity to bind the polysaccharide. Although relatively large quantities of deoxyribonucleic acid (DNA) modified the binding of polysaccharide to plasma protein *in vitro*, the injection of DNA simultaneously with labeled polysaccharide did not alter the effect of the latter upon the lymphosarcoma. Corticosterone and cortisol did not modify actively growing tumors, but after advanced regression, tumor growth might be re-initiated with cortisol.

THE EFFECT OF THE STREPTOKINASE-PLASMINOGEN SYSTEM ON THE DEVELOPMENT OF LOCAL STREPTOCOCCAL INFECTIONS IN RABBIT SKIN. Robert I. Krasner and Joseph J. Jannach, U.S. Army Medical Center, Japan (406).

The lysis of fibrin *in vivo* and *in vitro* by streptokinase-activated plasminogen suggests that streptokinase contributes to streptococcal virulence by interfering with the formation of a localizing fibrin barrier. This hypothesis has been investigated in the present study. Streptococci were suspended in (a) saline, (b) streptokinase, (c) human plasma or plasminogen, and (d) a combination

of the last two substances. The suspensions were injected into rabbit skin and the resulting lesions compared by gross and histologic examination.

The virulence of 3 group A streptococcal strains was increased by a combination of streptokinase and plasma or streptokinase and plasminogen as demonstrated by measurements of the resulting lesions. Plasma or plasminogen alone also increased virulence but frequently to a lesser degree. Streptokinase alone was without effect on virulence. Histologic examination revealed that little fibrin was present during the early stages of infection when the gross manifestations were evident.

The virulence of a group D streptokinase-negative streptococcal strain was not increased by either plasma or plasminogen alone, whereas a combination of either of these substances with streptokinase increased the extent of the resulting lesions. Streptokinase alone also increased the virulence of the group D strain, but not to the same extent as when combined with either plasma or plasminogen.

These results provide evidence that streptokinase contributes to the virulence of streptococci in a manner as yet undetermined but is independent of its action on fibrin.

**CIRCULATORY CHANGES IN COLD INJURY OF THE MOUSE EAR: AN EXPERIMENTAL APPROACH TO THE PATHOGENESIS OF VASOSPASTIC TISSUE DAMAGE.**† J. P. Kulka,\* Harvard Medical School and Robert B. Brigham Hospital, Boston, Mass.

Vasospastic ischemia may be concerned in the pathogenesis of a variety of lesions, including those of psychosomatic, allergic, and rheumatic diseases, as well as those of cold injury. The purpose of the present study was to investigate the sequence of circulatory changes in this type of pathogenic mechanism, using as an experimental model the cold-induced gangrene which develops in the ears of mice kept individually caged at an ambient temperature of 3°C. The local alterations in the terminal vascular bed were observed *in vivo* and in histologic sections. They were also examined in whole mounts of the cleared ears in which the vessels had been fixed in their natural state by rapid-freeze termination of the intact animal followed by amputation of the frozen ears, and dehydration in the cold. Evans blue injected intravenously, prior to termination, served to demonstrate abnormal extravasation of plasma macromolecules as well as vascular stasis.

The local circulatory changes observed prior to the onset of gangrene were successively: (a) generalized vasoconstriction; (b) venular relaxation; (c) progressive venular and capillary stasis; (d) arterio-arterial and arterio-venous shunting. Cutaneous necrosis conformed to the region of stasis and not to the more widespread vasospasm.

These observations indicated that the extent of necrosis in experimental non-freezing cold injury was determined by a by-passing of the capillary beds rather than by a failure of arterial supply. The technique of intravascular injection of vital dyes followed by rapid-freeze fixation should prove valuable in determining whether a similar microcirculatory impairment is concerned in the pathogenesis of other inflammatory lesions.

**THE REPLICATION OF AGGREGATES OF TUMOR CELLS.**‡ Joseph Leighton,\* Richard L. Kalla, and James M. Turner, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

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From its inception, invasive carcinoma commonly presents a pattern of nests of neoplastic cells in a matrix of connective tissue. As the tumor grows and involves larger volumes of tissue, it becomes evident that mechanisms exist by which the nests give rise to other nests of cancer cells (replication of aggregates). We are studying several fundamental questions concerning the relationship of aggregate replication to tumor invasion. Is aggregate replication regulated from within the aggregate or modified by host factors?

Aggregate replication was observed *in vitro*, in matrix cultures of a line of "transformed" human cells (Strain D-189) in the complete absence of living stromal cells. Subsequently, cells were suspended in a thin plasma clot under agar to observe directly the growth and replication of aggregates. From enlarging spherical colonies a few ribbons of cells grew out in various directions. As primary ribbons lengthened, secondary ribbons appeared in geometric patterns as branches of the primary ribbons. These produced still newer ribbons. The final pattern was one of an elaborate crisscross of many ribbons of cells. Branching cords of cells produced a complex arborization when the same line was grown in frozen-thawed umbilical cord matrix cultures.

The geometric patterns did not appear to result from cells following stress lines provided by the matrix. Instead, the patterns appeared to indicate that groups of cells functioned as integrated units in the propagation of similar groups, and that their reproduction might have qualities of predictability and rhythmic periodicity.

TWO TYPES OF "ENDOTHELIAL" CELL IN THE HUMAN RENAL GLOMERULUS.†  
J. F. A. McManus\* and Stanley Kurtz, University of Alabama Medical Center, Birmingham, Ala.

Electron microscopy of human renal glomeruli, fixed in Palade's buffered osmium and sectioned at 200 to 250 Å after acrylic embedding, showed two types of cells within the *lamina densa*. The nuclear structure in these cells was of one type, but the cytoplasm appeared to be of two varieties: (1) Cells lining capillary lumens showed loose cytoplasmic structure poor in particles with reticulation or fenestration when in contact with the *lamina densa* (type 1). (2) Cells forming the interluminal cell aggregates (ILCA) were characterized by dense cytoplasm, rich in particles, and contained masses of electron-dense material resembling basement membrane apparently within the cell substance as well as at the borders (type 2).

Where a type 1 cell came into contact with a type 2 cell, a distinct cell margin could be recognized with a contrasting appearance of the cytoplasm of the two cell types. Similar appearances were found in other species. The implications of the two types of "endothelial" cell in glomerular structure and in pathology are discussed.

HISTOCHEMICAL STUDY OF BREAST NEOPLASM. P. J. Melnick,\* and Weldon K. Bullock, Veterans Administration Hospital, San Fernando, University of California Medical School, Los Angeles, Los Angeles County General Hospital and University of Southern California Medical School, Los Angeles, Calif.

A histochemical study of 18 examples of breast neoplasm revealed abundant esterase, lipase and succinic dehydrogenase in well differentiated adenocarcinomas, less in scirrhous carcinomas, and none in medullary carcinomas. Alkaline phosphatase was found in only the myoepithelial layer of well differentiated adenocarcinoma; it was also found in the stroma of medullary carcinoma, but not in the stroma of scirrhous carcinoma or adenocarcinoma. Metachromasia of the

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ground substance was found in areas of active epithelial proliferation. In normal breast lobules, the enzymes, as well as metachromatic ground substance, were increased in the second half of the ovarian cycle. Villee and Rosa and Velardo presented chemical and histochemical evidence that in estrogen-sensitive tissues, estrogen acts as a co-factor for iso-citric transhydrogenase. This increases the rate of conversion of adenosine triphosphate to adenosinediphosphate, thus releasing more energy for metabolic activities.

**STUDIES ON THE ROLE OF INFLAMMATION IN CARCINOGENESIS.**† Valy Menkin,\* Temple University School of Medicine, Philadelphia, Pa.

Recent studies have indicated the presence of a growth-promoting factor in inflammatory exudates which offers a reasonable explanation for the mechanism of repair in inflammation. Repeated injections of this factor induced marked hyperplastic responses. In the breast tissue of non-pregnant rabbits, the resulting picture resembled that seen in chronic cystic mastitis. The surrounding cutaneous structures were likewise affected. Besides considerable cutaneous epithelial hyperplasia with involvement of the hair follicles, the latter exhibited hyperkeratosis. The growth factor, after its administration for many months, induced a state of hyperplasia that tended to be irreversible following cessation of injections. Nevertheless, it failed to establish autonomous growth in the sense of a neoplasm. The combined administration, however, of the growth factor of exudates and a carcinogen, e.g., methylcholanthrene, accelerated the production of papillomas and, in a few instances, of squamous carcinoma. The growth factor appeared to act as a cocarcinogen. This may well be the role of inflammation in carcinogenesis; namely, via the growth factor acting as a cocarcinogen to either an exogenous or possibly an endogenous carcinogen liberated as a result of genetic susceptibility.

The growth factor was diffusible and as such was obtained in the concentrated diffusate of exudates, but it was absent in the diffusate of blood serum. Chromatographic studies indicated the presence of a peptide in the active fraction as well as nucleotides or related derivatives. Absorption studies with a Beckman DU spectrophotometer revealed a peak at 260 to 270 mμ. The diffusibility, heat stability, and spectrophotometric absorption studies were all compatible with the presence of a nucleotide. Enzymatic studies with either trypsin or ribonuclease inactivated biologic activity of the factor. The observations are consistent with the interpretation that the growth factor of exudates is a biologically active nucleopeptide.

**GLOMERULAR FIBRINOID: A DIFFERENTIATION OF TWO TYPES.** P. O'B. Montgomery\* and E. E. Muirhead,\* The University of Texas Southwestern Medical School, Dallas, Texas.

The nature of glomerular fibrinoid in a case of "systemic fibrinoid" has been characterized by a group of procedures designed to identify the presence of the following substances: neutral fats, fatty acids, phospholipids, proteins, cholesterol, amyloid, indole, free carbonyl groups, tyrosine, calcium, free aldehyde groups, potassium, and protein-bound sulfhydryl groups. The tinctorial nature of this material has been additionally characterized by the hematoxylin and eosin, the Mallory and the Masson stains. The presentation contrasts the tinctorial and histochemical patterns in this case with the patterns obtained by applying the same procedures to glomerular fibrinoid as seen in arteriolar necrosis of malignant hypertension, and to two experimentally produced forms of glomerular

† Aided by a grant from the National Cancer Institute, United States Public Health Service.



fibrinoid. The first experimental type was produced by injecting partially autolysed smooth muscle into the renal vasculature of the dog; the second was produced by injecting fibrin into the renal vasculature of the dog. The comparison of histochemical and tinctorial patterns indicated that there were at least two types of glomerular fibrinoid and suggested a derivation from fibrin in one and altered smooth muscle in the other.

**THE FINE STRUCTURE OF THE GLOMERULUS IN AMYLOIDOSIS.** Henry Z. Movat,\*  
University of Toronto, Toronto, Canada.

Amyloid is believed by some to be deposited between the endothelium and the basement membrane of the glomerulus. Another view is that amyloid results in swelling or degeneration of the basement membrane, rather than a deposit of adventitious material. The latter opinion has been expressed also in a recent electron microscopic study.

Animal tissue was obtained by needle biopsy and examined by light and electron microscopy. In the former, impregnation with silver methenamine was particularly informative, showing an intact basement membrane with amyloid on either side of it. Thin sections of tissue fixed in osmium tetroxide were floated on either silver methenamine or silver albumose and examined with the electron microscope. The electron micrographs showed that the basement membrane remained intact in amyloidosis although the amyloid was deposited on either side of it; i.e., between endothelium and basement membrane, and basement membrane and glomerular epithelium. The epithelium showed vacuolization and partial loss or flattening of the foot processes. The basement membrane in areas with no amyloid deposit was thickened.

These observations indicate that glomerular amyloidosis is not an alteration of the basement membrane but a deposit, not only between endothelium and basement membrane, but on either side of the latter.

**STRONGER AND MORE SELECTIVE STAINING OF HISTOPLASMA BY SCHIFF'S REAGENT AFTER CONSECUTIVE OXIDATION BY PERIODIC AND CHROMIC ACID.**† Robert W. Mowry\* and Glenn S. Hooper, University of Alabama Medical Center, Birmingham, Ala.

Compared with other fungi, *Histoplasma* are often difficult to detect in sections or smears stained by the usual periodic acid-Schiff (PAS) method. Organisms are poorly colored, and staining of the caseous or fibrous background hinders their detection. With the Gridley stain, contrast is much better, but *Histoplasma* are seldom strongly colored.

*Histoplasma* were colored far more deeply by Schiff's reagent with simple modifications in the use of periodic acid. Cell walls of *Histoplasma* were more strongly colored if sections or smears were oxidized by either of two methods: (a) by aqueous (0.5 per cent) periodic acid for 60 minutes instead of the customary 5 to 10 minutes; (b) by treatment for 10 minutes in 0.5 per cent periodic acid dissolved in glacial acetic acid instead of water. In order to lighten the staining of the tissue background by Schiff's reagent, sections were then washed and further oxidized for 30 to 45 minutes in 5 per cent chromic acid. After washing, sections were subjected to the usual treatment in Schiff's reagent, including bisulfite rinses. Further contrast of organisms against tissue resulted from counterstaining in metanil yellow, as prescribed in the Gridley

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stain. Hematoxylin was also used for nuclear staining. In the final result, cell walls of Histoplasma were deeply colored by Schiff's reagent while the tissue background was orange to yellow. This method produced better results than either the conventional PAS or the Gridley stain. After oxidation in both periodic acid and chromic acid, alternate sections can be treated if desired in methenamine silver instead of Schiff's reagent.

**THE APPLICATION OF PARABIOSIS FOR THE STUDY OF VALVULAR ENDOCARDIAL LESIONS.**† K. Nakao, M. Oka, and A. Angrist,\* Albert Einstein College of Medicine, New York, N.Y.

The stress of parabiosis has been used in the experimental production of valvular lesions in rats. Simple parabiosis yielded edematous swelling (52 per cent), increased cellularity (43 per cent), alterations in collagen (19 per cent) and vegetations (11 per cent) in the mitral valve; the tricuspid showed somewhat lower incidence. Parabiosis with one partner rendered resistant to Novikoff hepatoma by regression of an intramuscular transplant showed more marked alterations characterized by more prominent cellularity, collagen alteration, and fibrinoid; the tricuspid in this instance had a higher incidence of such involvement. The charted observations in these groups are compared.

Parabiosis was utilized to obtain an intrinsic hormonal imbalance by ablating endocrine organs with known feed-back effect on their pituitary tropic controlling hormones. Castration in one parabiont yielded more marked alterations, as edema (89 per cent), increased cellularity (67 per cent), changes in collagen (25 per cent) and fibrinoid (11 per cent) in the mitral valve; the tricuspid exhibited fibrinoid, collagen distortion and vegetations with a higher incidence. The lesions were present in both castrates and intact animals. Adrenalectomy in one parabiont was accompanied by more marked alterations than simple parabiosis of intact rats. Estrogen (35 to 50 mg. in 5 weeks to 200 to 250 gm. males) preliminary to parabiosis resulted in the highest incidence and the severest lesions.

**DIAGNOSIS OF ENDOMETRIAL LESIONS BY CYTOMETRIC EVALUATION OF ASPIRATED CELLS.** H. E. Nieburgs, The Mount Sinai Hospital, New York, N.Y.

The evaluation of endometrial cell changes in cytologic specimens obtained by aspiration from the uterine cavity is often difficult. There is an overlapping in the size of the nuclei of cells obtained in the secretory phase, glandular hyperplasia, polypoid hyperplasia, placental tissue, and in carcinoma. The visual integration of the amount and heterogeneity of nuclear material to the diameter of the nucleus is often not possible. An attempt was therefore made to carry out linear measurements of all nuclear particles with a specially designed planimeter and to relate the total linear diameter of the particles to the linear diameter of the nucleus. This was carried out on polaroid photographs taken at a magnification of 2,000. The percentage of the total diameter of all nuclear particles in relation to the nuclear diameter is expressed as the karyo-heterogenic index (KHI). It was found that the KHI for non-malignant cells ranged between 50 and 100, while in adenocarcinoma, a KHI range of 114 to 190 was found. In most cases, however, the lowest measurement recorded for malignant endometrial cells was 125. The lower KHI between 125 and 114 recorded for cells in adenocarcinoma was apparently due to postexfoliative deterioration of cells.

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THE ACTIVITY OF SUCCINIC DEHYDROGENASE IN THE EXPERIMENTAL EPENDYMOMA OF C<sub>3</sub>H MICE.† Kazuo Ogawa and H. M. Zimmerman,\* Montefiore Hospital, New York, N.Y.

Transplantable ependymomas induced by methylcholanthrene in C<sub>3</sub>H mice were used. Succinic dehydrogenase (SD) activity was studied by biochemical (Schneider and Potter's manometric method) and histochemical (modified Goddard and Seligman's method) methods under aerobic conditions. Mitochondria were identified by Mallory's ferric chloride oxidation followed by the phosphotungstic acid hematoxylin stain. Normal C<sub>3</sub>H mouse cerebrum was used as a control.

The present investigation revealed: (1) SD activity in the ependymoma was much lower than in the normal cerebrum ( $QO_2$ :  $0.69 \pm 0.302$   $\mu$ l. to  $2.24 \pm 0.318$   $\mu$ l.). (2) In normal cerebrum as well as in ependymoma, there was a close parallel between the amount, distribution and localization of mitochondria and SD activity. (3) Neurons revealed highest SD activity, followed by choroidal epithelium and ependymal cells. Formazan granules were scattered throughout the neuropil. Oligodendrocytes, particularly satellites, showed moderate activity. Occasionally, very low activity was present in astroglia. (4) Ependymoma cells contained a smaller amount of formazan granules than average normal cerebrum, and the mitochondrial dots, rods, and filaments in ependymoma cells were smaller, finer and fewer. In addition, ependymoma cells in mitosis, particularly in the prophase and metaphase, appeared to have lower SD activity and to be poorer in mitochondria than cells in the interphase.

It is postulated that the low enzymatic activity observed in ependymoma is related to (a) the scarcity of mitochondria; and (b) the high incidence of mitotic cells.

PRODUCTION OF INTRAVASCULAR CLOTting IN THE RABBIT BY INTRAVENOUS INJECTION OF BLOOD THROMBOPLASTIN. H. C. Pirkle, H. C. Anderson, J. J. McHugh and V. G. Allen, University of Louisville School of Medicine, Louisville, Ky., and Massachusetts General Hospital, Boston, Mass.

Each of 8 rabbits received injections of 2 ml. of a human thromboplastin generation test incubation mixture (aged serum, Al(OH)<sub>3</sub>-adsorbed plasma, CaCl<sub>2</sub>, Asolectin) at its peak activity. Six animals developed seizures within 1 minute and died within 2 to 10 minutes after injection. Two animals showed no untoward signs. Grossly visible clots in the right heart and pulmonary arteries and microscopic plugging of small arteries by fibrin were found in all animals. Post-mortem clotting was prevented by heavily heparinizing the animals before death.

In one control group, each of 4 pairs of rabbits received different single components of the experimental mixture. A fifth pair received injection of bovine thrombin equivalent to that which developed in the experimental mixture. No animal in this group showed gross or histologic evidence of intravascular clotting.

In a second type of control procedure, each of 4 pairs were given injections of incomplete incubation mixtures in which saline containing a trace of thrombin (equivalent to that in the experimental mixture) was substituted in turn for one of the 4 constituents of the thromboplastin generating system. None of these animals exhibited untoward signs or gross indications of intravascular clotting. However, a rare microscopic clot could be found in the lungs of some

† This investigation was supported by a grant (No. 73-4) from the National Multiple Sclerosis Society.

of the animals receiving mixtures possessing feeble thromboplastic activity.

We have also produced lethal intravascular clotting by injecting ultracentrifugally separated blood thromboplastin generated in a fresh serum-platelet system.

**ANGIOMA-LIKE LESIONS OF ADRENAL GLANDS.** A. Plaut,\* Armed Forces Institute of Pathology, Washington, D.C.

Adrenal circulation in man and other mammals presents a number of unsolved problems in structure and function. Some of these problems appear to be reflected in tumors and tumor-like lesions of adrenal vessels. There is no sharp border line between angiectasia and angioma, and the word "angioma" is used here loosely. Thirty "hemangiomas" were studied. They were unilateral, mostly confined within the capsule of the organ, and were found accidentally at necropsy. There was no relationship to endocrine disorders or other clinical syndromes, and they were found in traumatic death of healthy soldiers.

The cavernoma type prevailed, with lesions consisting of groups of distended capillaries or deep red nodules as much as 2 cm. in diameter. Even typical cavernomas might show endothelial overgrowth and capillary budding. Truly neoplastic lesions occasionally prevailed. Fibrosis was more frequent in older people. In some instances the fibrosis in the neighborhood of the tumor appeared to be a concomitant phenomenon if not a precursor.

Cases of diffuse phlebectasia with or without participation of extracapsular veins were of special interest. One might assume that disturbances of venous outflow were the basis of such lesions. The propelling or retaining action of the musculature of the adrenal vein, the direction of the bloodstream in the innumerable intra-adrenal anastomoses, and the amount of outflow through extracapsular veins are unknown factors.

There were also 3 noncystic lymphangiomas, 2 of them locally destructive.

**THE PANCREATIC DUCTAL SYSTEM IN MAN; A STUDY UTILIZING VINYL-ACETATE CASTS.** John T. Prior,\* Leon G. Berman, Stanley Abramow and Donald Ziegler, State University of New York, Upstate Medical Center, Syracuse, N.Y.

Considerable controversy exists regarding the normal anatomy of the human pancreatic ductal system, and also the possible role of this system in the pathogenesis of pancreatitis. To date, there has not been available a technique for demonstrating this ductal system *in vivo* in a manner comparable to cholangiography or urography.

The present study utilized injections of vinyl-acetate and latex into 144 human pancreases obtained from routine necropsy material. The age distribution ranged from 10 months to 90 years, and a variety of disease states were encountered; i.e., gall bladder and liver disease, diabetes mellitus, pancreatitis and pancreatic neoplasms.

The ductal system was outlined with the plastic substances, using the duct of Wirsung, the common bile duct and the main duct in the pancreatic tail as portals of entry. Blocks of tissue were removed for subsequent microscopic examination before the injected specimen was digested in potassium hydroxide or hydrochloric acid, or cleared according to the alcohol-methyl-salicylate technique.

The results of this investigation disclosed a wide range of variation in the arrangement and degree of development of the primary, secondary and tertiary ductal branches. Particular emphasis was placed upon the relationship of the duct of Wirsung to the ampulla of Vater, the Santorini system and its range of normal development. The presentation includes comments on the common channel, the pancreatic capsule and the possible role of ductal disease in the etiology of pancreatitis.

**PLOIDY OF PRIMARY AND METASTATIC HUMAN TUMORS.** Giancarlo Rabotti, National Cancer Institute, Bethesda, Md.

The content of Feulgen-stained deoxyribonucleic acid (DNA) in interphase nuclei of cells of certain primary human tumors was compared with the nuclear Feulgen stained DNA content in their metastases. The quantitative measurement of the DNA carried out by microspectrophotometric technique on single nuclei stained with the Feulgen reaction has been established as a suitable method for the evaluation of the nuclear chromosomal set. The human tumors studied were 4 examples of breast adenocarcinoma with lymph node metastasis; one instance of breast carcinoma with metastasis in liver and bone (skull); and one case of carcinoma of the nasopharynx with liver and adrenal metastases.

The cells in the metastatic lesions, when compared with those of the tumor of origin, were deficient in the diploid class nuclei. No significant differences between the ploidy of the metastatic lesions were observed in different organs of the same subject.

The nuclear volumes of tumor cells were significantly greater in the metastatic lesions, and the distributions of the volume estimations had a wider spread than the values found in the primary tumor. On the basis of these data, the possibility of a cytogenetic basis for the phenomenon of metastasis is discussed.

**OBSERVATIONS ON THE GROSS ANATOMY, CORONARY ARTERIES AND MYOCARDIUM OF A LARGE WHALE HEART (256 POUNDS).** George J. Race,\* W. L. Jack Edwards, E. R. Halden, Hugh E. Wilson, and Francis J. Luibel, The University of Texas Southwestern Medical School, Dallas, Texas.

A large whale heart from an adult male sperm whale (*Physeter catodon*) was secured in the fresh state. The animal weighed approximately 47,700 pounds. Careful dissection of the coronary arteries and venous system was made. Heart chambers and valves were similar to those of other mammals. The coronary arteries had extremely large right and left marginal branches which supplied the major lateral mass of the right and left ventricles. The venous system was similar to that in other mammals. Crude ventricular volume (calculated by means of the formula of a hemi-ellipsoid) and cardiac output were calculated. The latter was found to be 453 liters per minute based on a rate of 10 per minute, assuming complete emptying. Cardiac muscle fiber was similar in size to human myocardial fiber except in a fetal whale heart (weight, 1,600 gm.) where very small fibers were found. The aorta was found to be 20 cm. in diameter and the wall to consist of very large interwoven bundles of elastic tissue and fibrous tissue apparently devoid of muscle. There were no gross or microscopic evidences of arteriosclerosis. Comparative estimated cardiac output: body-weight ratios and heart-weight:body-weight ratios were made between the whale and the human subject. The heart-weight:body-weight ratio was similar, being .47 per cent in the adult whale and .43 per cent in the average human subject. The cardiac output:body-weight ratio was somewhat less in the adult male whale, being 2.1 per cent as compared with 5.3 per cent in the adult human being.

**FRACTIONATION OF ANTI-A AND ANTI-B ISOHEMAGGLUTININS BY ANION-CATION CELLULOSE EXCHANGE CHROMATOGRAPHY.** Arnold J. Rawson\* and Neva M. Abelson, Hospital of the University of Pennsylvania, Philadelphia, Pa.

Through the use of an anion exchange adsorbent, N,N-diethyl-aminoethyl-cellulose (DEAE-cellulose), and a cation exchange adsorbent, carboxy-methyl-cellulose (CM-cellulose), isohemagglutinins anti-A and anti-B were separated into 3 components. One fraction, of low anionic binding capacity, was associated

with gamma-2 globulins of low sedimentation constant. Its serologic behavior was characteristic of the "immune" antibody. A second fraction was of intermediate anionic binding capacity. It consisted of an albumin-globulin mixture whose most conspicuous globulin component was alpha-2. The sedimentation constant (uncorrected) of the non-albumin portion of this fraction was found to be 15.2. A third fraction was of high anionic binding capacity. It contained some non-gamma globulin and serologically active gamma-1 globulin. This gamma globulin was not homogeneous with respect to the sedimentation constant. Serologically, the second and third fractions were similar in that they were not enhanced by high-protein diluents, but the third fraction contained relatively more hemolysin activity than the second.

A HISTOCHEMICAL STUDY OF OXIDATIVE ENZYMES IN THE SCIATIC NERVE OF THE RABBIT. Flaviu C. A. Romanul and Richard B. Cohen, Massachusetts General Hospital, Boston, Mass.

The sciatic nerves of 20 male and female albino rabbits were submitted to a series of histochemical reactions for the demonstration of oxidative enzymes. Particular emphasis was placed on reactions which demonstrated succinic dehydrogenase, DPNH diaphorase and TPNH diaphorase. By means of a cryostat, longitudinal and transverse sections of variable thickness were made along the course of each nerve. The sections were allowed to incubate in substrate solution for varying periods of time in order to emphasize particular biochemical and histologic features.

Succinic dehydrogenase activity was most intense in 4 to 6 thin longitudinally arranged lines on either side of each node of Ranvier. The nodes of Ranvier were thus demonstrated in bold relief against the less active elements of the nerve. In cross sections it was evident that the enzyme activity was most concentrated in thickenings of the Schwann cell cytoplasm in the region of the node, and that the endoneurium and the myelin sheath showed no activity. The perikaryon of the Schwann cell stained less intensely. Slight activity was observed in the axon.

In contrast, DPNH and TPNH diaphorase activity was most intense in the region of the perikaryon of the Schwann cell.

The correlation of these studies with data obtained by conventional histologic technique, electron microscopy and quantitative chemistry, and the significance of the observations from the neuropathologic and biochemical points of view are discussed.

THE PATHOLOGY OF EXPERIMENTAL MOUSE VIRUS HEPATITIS. B. Reubner and J. L. Bramhall, Dalhousie University, Halifax, N.S., Canada.

Groups of 30 weanling mice were infected with 0.1 ml. of a 10 per cent liver suspension containing M.H.V.<sub>3</sub> virus. The mice were free of *Eperythrozoon coccoides*, which enhances the hepatitis. The effect of injection by various routes was compared.

After intraperitoneal inoculation, the mortality was always more than twice that observed in mice infected by the subcutaneous route. This difference was statistically significant. The maximum mortality occurred on the fifth day after intraperitoneal injection and on the seventh or eighth day after subcutaneous injection. The mortality after intravenous injection was intermediate between that of the intraperitoneal and subcutaneous routes.

Histologic examination showed that with intraperitoneal injection, the first foci of necrosis appeared on the surface of the liver after 48 hours. During the next few days, lesions developed in the substance of the liver. The process

seemed to spread mainly along centrilobular veins. The first alteration seen in the damaged cells was nuclear swelling followed by karyorrhexis. The cytoplasm lost its normal basophilia, and eosinophilic necrosis developed. Soon afterwards, inflammatory cells, mainly neutrophils, infiltrated the lesion. During the hepatitis, the hepatic glycogen was depleted and fatty alteration developed. The reticulin network was damaged. The lesion resembled that produced by a necrogenic diet in rats. The virus also produced necrosis in retroperitoneal fat, abdominal lymph nodes and spleen.

After subcutaneous injection, lesions in the dermal fat occurred within 24 hours. Liver lesions appeared about 2 days later than after intraperitoneal injection and differed in distribution. Scattered foci of necrosis developed simultaneously. After intravenous injection, the liver lesions resembled those produced by subcutaneous injection but tended to occur earlier.

**ELECTRON MICROSCOPIC STUDY OF HUMAN CHOLESTASIS.** Fenton Schaffner and Hans Popper,\* The Mount Sinai Hospital, New York, N.Y.

In order to investigate the pathogenesis of intrahepatic cholestasis, liver biopsy specimens from patients with drug-induced intrahepatic cholestasis were examined under the electron microscope. For comparison, specimens from patients with extrahepatic biliary obstruction, the Dubin-Johnson syndrome, and viral hepatitis were similarly examined. In confirmation of the observations with light microscopy, bile canaliculi were found to be conspicuously dilated in extrahepatic biliary obstruction and normal in the Dubin-Johnson syndrome. They were frequently not dilated in cases of intrahepatic cholestasis. Biliary microvilli were absent in extrahepatic biliary obstruction and were reduced and distorted in intrahepatic cholestasis even in undilated bile canaliculi. They were normal in cases with the Dubin-Johnson syndrome. In the latter condition, the pigment granules differed in their electron microscopic appearance from bile as seen in bile plugs, and were also distinguishable from what were presumed to be lipofuscin and fat droplets. Observations made thus far, as correlated with functional and conventional microscopic features, suggest that intrahepatic cholestasis represents a primary defect localized in the canalicular microvilli, which results in altered permeability of the canaliculi for either water or proteins. The same defect may be a secondary result of mechanical hydrohepatosis. In contrast, in the Dubin-Johnson syndrome, the defect is solely in the transfer of bilirubin and some dyes. Further investigations are being directed to the alteration of mitochondria and other cellular details in the conditions studied.

**TRANSPLANTATION OF MALIGNANT NEOPLASTIC CELLS FROM TISSUE CULTURE TO RAT BRAINS.** Thomas M. Scotti,\* Martha A. Wryk, Mantley Dorsey, Jr., and M. Michael Sigel, University of Miami School of Medicine and Variety Children's Hospital, Miami, Fla.

Several lines of established cultures of epidermoid carcinoma cells were found to produce tumors upon inoculation into the brains of rats. Considerable growth was obtained with KB cells and with one line of HeLa cells. Another line of HeLa cells, with few exceptions, produced only minimal to slight growth. The brains were examined periodically up to 18 days after inoculation. Although some growth was noted at 4 days, the maximum generally was obtained at 11 to 15 days. Growth was present in the brain, in the ventricles, and in the meninges, not only at the sites of inoculation but in other portions of the brain. In most experiments, the animals were conditioned by means of cortisone and x-radiation. In one experiment, however, it was found that a significant growth was obtainable following x-radiation treatment alone. Poliomyelitis virus multiplied



in brains bearing tumors derived from HeLa and KB cells, but not in brains free of tumor cells.

**PULMONARY *Pneumocystis carinii* INFECTION IN RABBITS.** Walter H. Sheldon,\* Emory University School of Medicine, Atlanta, Ga.

Many organisms morphologically identical with those encountered in infants with *Pneumocystis pneumonia* and moderate to marked interstitial pneumonitis were found in 14 rabbits treated for 6 to 33 days with massive doses of cortisone, penicillin and streptomycin after intranasal instillation of a lung tissue suspension from a patient with widespread *Pneumocystis pneumonia*. Similar lesions with numerous *Pneumocystis* organisms were present in 6 rabbits treated in the same manner and inoculated with a lung tissue suspension from a rabbit previously inoculated with infected human lung. The experimental lesions did not reproduce the massive consolidation of *Pneumocystis pneumonia*, but resembled the subclinical form of *Pneumocystis pneumonitis* in man reported from this laboratory.

Control experiments devised to test the transmission of the infection revealed interstitial pneumonitis and *Pneumocystis* organisms of similar nature and frequency to those described above in 12 rabbits receiving cortisone, antibiotic agents and either a boiled suspension of normal human lung or saline. The administration of only antibiotic agents and infected rabbit lung suspension to 9 rabbits produced less marked interstitial pneumonitis with fewer organisms. However, rare *Pneumocystis* organisms and minimal focal pneumonitis were found in 11 of 13 rabbits which had received neither cortisone, antibiotic agents nor any inoculum.

The observations did not prove the transmission of the infection to rabbits but showed that latent *Pneumocystis* infection is widespread in these animals. Moreover, the change from a latent infection to *Pneumocystis pneumonitis* appeared to depend upon an impairment of host resistance produced most effectively in these experiments by the combined administration of cortisone and antibiotic agents.

**HOST FACTORS IN CARCINOMA OF THE COLON.** Sheldon C. Sommers,\* Massachusetts Memorial Hospitals, Boston, Mass.

The clinical records and necropsy observations in over 450 cases of carcinoma of the large intestine have been investigated. Particular attention was given to the constitutional aspects. Family histories of cancer or diabetes, and clinical or pathologic evidences of obesity, hypertension, arteriolar nephrosclerosis, arteriosclerotic heart disease and diabetes were not uncommon. Some endocrine abnormalities were found in about 90 per cent of the cancer cases, but were unusual in the cancer age group in less than half of the individuals with cancer of the colon, as compared to matched noncancerous control cases. This suggested that other factors such as heredity and environmental carcinogens were frequently important. Benign gastric and colonic polyps and fatty infiltration of the pancreas were more common in the colonic cancer group than in the controls. Genital hyperplasia or hypertrophy, ascribable partly to estrogenic stimulation, occurred in about half of the cases of colonic carcinoma; the genital system was the other most frequent site of multiple primary cancers aside from the large intestine. A minority of colonic carcinoma cases had visceromegaly, unusually heavy bone structure, or other alterations ascribable to endocrine activity, thought to reflect the effects of pituitary growth hormone. Pituitary cell counts and other data have been analyzed regarding the significance of endocrine and other factors in the development and growth of colonic carcinomas.

**FURTHER STUDIES ON THE ULTRA STRUCTURE OF THE KIDNEY IN THE NEPHROTIC SYNDROME.** David Spiro, Massachusetts General Hospital, Boston, Mass.

Evidence that the basic structural lesion in the nephrotic syndrome consisted of defects in the glomerular capillary basement membrane are briefly reviewed.

The proximal convoluted tubular epithelium from such patients contained numerous, dense, intracytoplasmic "colloid droplets" which were either limited to the membrane or situated within small vacuoles. The tubular basement membranes underlying such cells were frequently reduplicated, split or of cribriform nature.

Similar intracytoplasmic colloid droplets were noted in connective tissue cells, lying adjacent to the proximal convoluted tubules as well as in the endothelial cells of the peritubular capillaries. It seemed probable that the existence of these intracellular particles in epithelial, mesenchymal and endothelial cells reflected the pathway of partial protein resorption known to occur in the nephrotic syndrome.

Colloid droplets were also formed in the confluent epithelium and the endothelium of the glomeruli from nephrotic patients. This suggested that the confluence of epithelial cell foot processes associated with proteinuria might in part reflect an attempt to reabsorb protein from Bowman's space itself.

**CERTAIN DETERMINING FACTORS IN THE PRODUCTION OF NEOPLASM IN RATS WITH PARADIMETHYLAMINOAZOBENZENE (BUTTER YELLOW).** Bernhard Steinberg,\* Toledo Hospital Institute of Medical Research, Toledo, Ohio.

Albino rats from a stock colony bred for the last 25 years were fed paradimethylaminoazobenzene, 0.6 per cent, in unpolished rice with 1 gm. of carrot per rat per day. Of 134 rats in the experiment, several groupings were made. One grouping was determined by the time of exposure to the chemical, which varied from 1 to 8 weeks. The second grouping was made according to the age of the animal (1 to 3 months and 6 to 24 months) at the time of exposure. A third grouping was based on the type of diet after exposure to the chemical was completed. Diets consisted of high fat, high protein and the usual stock ration. Many investigators have demonstrated the development of liver cancer in rats following feeding of butter yellow and a protein-deficient diet over long periods. In this study, minimum exposure to the chemical resulted in the development of leukemia and neoplasms of several types in several organs after the animals reached a certain age. Dormancy of the affected tissue combined with the "age factor" appeared as predisposing conditions to leukemia and neoplasm. The type of diet after exposure to the chemical appeared to be another determining condition in the development of the lesions. An excessively high fat diet was associated with an increased incidence of these conditions. Animals fed with a high protein diet fared best, and those on the usual stock ration had a moderate incidence of neoplasm or leukemia.

**HISTOCHEMICAL, HISTOLOGIC AND ELECTRON MICROSCOPIC OBSERVATIONS ON JEJUNAL EPITHELIUM IN NON-TROPICAL SPRUE.**† Elliott W. Strauss, Helen A. Padykula, Aaron J. Ladman and Frank H. Gardner, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.

Jejunal mucosa was obtained with the Crosby-Kugler intestinal biopsy device from 7 patients with non-tropical sprue and from 18 "hospital" control subjects.

Normal crypt cell cytoplasm exhibited intense basophilia but displayed minimal acidophilia after staining with eosin-methylene blue, and there was a

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paucity of enzymes (alkaline and acid phosphatase, esterase, adenosine triphosphatase, succinic dehydrogenase). Villous cell cytoplasm was acidophilic but contained some apical ribonucleic acid; intense cytoplasmic activity of all enzymes was present.

In sprue, crypts were deeper, villi were almost absent, and mitotic figures were more numerous, particularly in the crypts. Crypt cells appeared normal histochemically, but there was more enzymatic activity in a zone between the crypt and the surface epithelium. The latter had increased basophilia, little enzymatic activity and acidophilia. Electron microscopy of surface epithelium revealed irregular, shortened microvilli, many abnormal mitochondria, and in 3 of 4 patients with sprue, abundant RNA particles in apical cytoplasm unassociated with membranes of endoplasmic reticulum. In 4 controls, this RNA particulate disposition was unobserved.

In normal intestine, crypts are the germinative sites for columnar cells which ascend the villi. In sprue, cell production sites may be shifting since occasional mitotic figures were observed in surface cells.

Histochemical and ultrastructural observations suggested that in sprue, surface cells were undergoing a modulation of form and biochemical nature different from normal villous cells, and although the number of mitotic figures was increased, suggesting active proliferation, this did not compensate for the process producing the abnormal villi.

**PATHOLOGY OF SCHISTOSOMIASIS IN AFRICAN BABOONS.** Jack P. Strong,\* Henry C. McGill, Jr.,\* Joseph H. Miller and Jack C. Geer, Louisiana State University School of Medicine, New Orleans, La.

Necropsies were performed on 150 baboons (*Papio doguera*) immediately after they were trapped in Kenya, East Africa. These animals were found to have a variety of parasitic infections as well as fat-containing aortic intimal lesions. Of particular interest was a high prevalence of infection with *Schistosoma mansoni*. Fecal specimens of a few of the animals contained ova. Lesions related to schistosomiasis included numerous, minute, superficial ulcerations in the colon, and pseudotubercles containing ova in the mucosa and submucosa of the colon and in the liver. In addition, ova were present in the mucosa of the colon and, rarely, in the ileum, without significant tissue reaction. Adult worms were occasionally found in portal vein branches in histologic sections of the liver.

This is the first reported natural infection with *Schistosoma mansoni* in primates other than man in Africa. This observation is significant with respect to the control of human schistosomiasis in endemic areas where baboons are numerous.

**THE PATHOGENESIS OF EXPERIMENTALLY INDUCED MURINE LYMPHOBLASTOMA.** Paul B. Szanto,\* Steven O. Schwartz, H. M. Schoolman and Wilma Spurrier, The Hektoen Institute for Medical Research of Cook County Hospital, Chicago, Ill.

C3Heb mice were inoculated intracerebrally (i.c.) or intraperitoneally (i.p.) with cell-free filtrates prepared from a pool of leukemic C3Heb mouse brains. Another group of C3Heb mice received a tumor cell suspension i.p. For controls, cell-free filtrates prepared from brains of non-leukemic C3Heb mice were injected i.p. and i.c. into comparable animals. Swiss mice were inoculated i.c. with cell-free filtrates prepared from the brains of leukemic Swiss mice. Two animals were sacrificed each day from each group for histologic examination. All the animals except those in the control group developed lymphoma 7 to 12 days after inoculation.

The purpose of this study was to demonstrate the location of the earliest alterations and the differences in the distribution of the later changes in the lymphomatous animals. The earliest lesions appeared in the perigenital fat in the C<sub>3</sub>Heb animals 3 days after inoculation, regardless of the route of administration or whether the inoculum was brain filtrate or tumor cell suspension. In the Swiss mice, the earliest lesions were in the perirenal fat tissue. The lymph nodes, spleen, liver, and thymus were unaffected at this point.

The lesions were definite 4 days after inoculation. Tumor cell infiltration at this time was localized to the fat tissue around the kidneys and genital organs. The hepatic lesions were more prominent and appeared earlier in the Swiss mice than in the C<sub>3</sub>Heb mice. Five days after inoculation, lymph node involvement was definite and the various lymph nodes were affected simultaneously.

**A FLUORESCENT STAINING METHOD FOR DEMONSTRATING AMYLOID.** Philip S. Vassar, Charles F. A. Culling and H. E. Taylor,\* Faculty of Medicine, University of British Columbia, Vancouver, B.C., Canada.

Investigation of a number of fluorescent staining methods using an ultra-violet light source has led to the observation that amyloid in conventionally prepared, formalin-fixed paraffin sections, gives a specific bright yellow fluorescence after staining for 3 minutes with a 1 per cent aqueous solution of the dye thioflavine T, followed by differentiation for 10 minutes with 1 per cent acetic acid. Control sections of diabetic glomerulosclerosis, arteriolonephrosclerosis, membranous and chronic glomerulonephritis, many normal tissues, and different types of hyaline degeneration were also examined with this technique and proved to be negative. It was found that only primary and secondary amyloidosis gave this specific reaction. A positive result was obtained in all such cases examined, and brilliant fluorescence was demonstrable in areas where suspected amyloid gave inconclusive staining reactions with methyl violet or congo red. The staining method is rapid, easily performed, and inexpensive. It has been adopted as the method of choice for demonstrating amyloid in our laboratory. The observations in an interesting case of diffuse primary amyloidosis as well as representative tissue from cases of secondary amyloidosis are presented.

**ENZYMATIC HISTOCHEMISTRY OF THE POTASSIUM-DEFICIENT RAT KIDNEY.**† Max Wachstein\* and Elizabeth Meisel, St. Catherine's Hospital, Brooklyn, N.Y.

Various enzymatic staining reactions were applied to the kidneys of rats on potassium and potassium-sodium deficient diets. Techniques used included those for alkaline and acid phosphatase, adenosine triphosphatase, esterase, succinic dehydrogenase, DPNH and TPNH diaphorase. With the exception of succinic dehydrogenase, all procedures were carried out in frozen sections of tissue fixed in cold Baker's formalin. This guaranteed an adequate state of tissue preservation at a cytologic level particularly valuable for the evaluation of DPNH and TPNH diaphorase activity.

Alterations were most marked in animals on the doubly deficient diet. Significant increases of acid phosphatase and esterase were seen not only in the collecting ducts and macrophages in the medulla, but also in the thin limbs of Henle's loops. The PAS-positive granular deposits in collecting ducts, which were regularly seen in the potassium-deficient rat kidney, reacted in positive manner for acid phosphatase and esterase. The cell membranes of the collecting ducts showed a distinct increase in adenosine triphosphatase activity. The intercalated cells of the median portions of the collecting ducts were strongly stained with the various dehydrogenase techniques. The increase in their number in

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the deficient kidney was reflected in the enzyme preparations. While in the initial stages of the deficiency only inconsistent alterations occurred in the cortical tubules, the ascending limbs of Henle's loops revealed a uniform decrease of various enzymatic staining reactions. This decrease appeared to coincide with the moderate dilatation of the ascending limbs caused by the blocking effect of the proliferating cells in the collecting ducts. The increase in enzyme activity in the tubules located in the inner medulla, on the other hand, was interpreted to indicate a state of increased functional activity aimed at overcoming the severe disturbance of the concentrating mechanism which characterizes experimental potassium deficiency.

**HISTOCHEMICAL STUDIES OF SUCCINIC DEHYDROGENASE IN PROLIFERATIVE LESIONS OF THE LARGE INTESTINE IN MAN.** Lee W. Wattenberg,\* University of Minnesota Medical School, Minneapolis, Minn.

A morphologic histochemical study of succinic dehydrogenase (tetrazolium salt reduction technique) has been carried out in proliferative lesions of the large intestine including regenerating mucosa, hyperplasia, adenomatous polyps, and carcinoma.

A marked decrease in staining for succinic dehydrogenase as compared with normal mucosa occurred in elements of all of the proliferative lesions. In well differentiated glandular forms, the more superficial portions showed low succinic dehydrogenase activity whereas deeper, a relatively higher activity existed. Thus, in hyperplasia, surface and crypt epithelium, except for the cells at the base of the crypts, gave a very weak reaction. The activity in the base of the crypts varied and was frequently quite intense. In polyps, a low succinic dehydrogenase activity was frequently observed in the surface epithelium and outer portion of the crypts of the atypical glands found in these lesions, whereas the deeper portion of these crypts stained intensely. In well differentiated carcinoma, epithelium with either low or high activity was found. The latter and some of the former appeared to represent malignant counterparts of comparably staining epithelium in the benign lesions. Weak staining also occurred with decreasing differentiation of intensely staining epithelium, even at an early stage of this process.

In proliferating lesions in which poorly differentiated forms occurred, a weak reaction for succinic dehydrogenase was observed throughout such epithelium. Accordingly, regenerating epithelium (2 to 8 days after production of a mucosal ulcer) stained weakly for the enzyme, and poorly differentiated carcinoma also showed low activity.

**HISTOCHEMICAL STUDIES OF THE PATHOGENESIS OF HUMAN CORONARY ARTERY ATHEROSCLEROSIS.** Harvey F. Watts, Temple University Medical School, Philadelphia, Pa.

Histochemical studies were performed on human coronary arteries from young and old, male and female patients with minimal to severe atherosclerosis, using formaldehyde-fixed tissues and fresh frozen sections. Emphasis was on the intimal ground substance alterations and their possible relationship to the lipid accumulations seen in atherosclerosis. Connective tissue, PAS, Ritter-Oleson and oil red O stains were performed. Toluidine blue stains controlled by hyaluronidase, beta-glucuronidase, and glucosidase enzymatic digestions were used to study metachromatic substance. Fluorescein-labeled antibody against human beta-lipoprotein was used to find the blood lipid carrier in the lesions.

The results were as follows: Beta-lipoprotein was detectable in atherosclerotic lesions in the earliest to the most advanced forms. It lay in the regions of extracellular lipid accumulations and in some of the lipid-bearing macrophages.

Stainable beta-lipoprotein was associated with metachromatic intimal ground substances. Not all metachromatic areas were stainable for beta-lipoprotein, but nearly all beta-lipoprotein reactions occurred in metachromatic areas. Advanced fibrotic lesions, where metachromasia was lost also might show stainable beta-lipoprotein. Hyaluronidase and beta-glucuronidase removed the metachromasia from the beta-lipoprotein stainable areas.

Thus, in the human coronary artery intima, an intimate interrelationship was demonstrated among hyaluronidase and beta-glucuronidase labile metachromatic ground substances, plasma beta-lipoprotein, and accumulated lipid material in all stages of atherosclerotic lesions. The pathogenic implications of these observations are discussed.

**RADIOAUTOGRAPHY OF THE ISOLATED NEPHRON.** B. T. Williams and J. P. Wyatt,\* St. Louis University School of Medicine, St. Louis, Mo.

One of the best techniques for investigating renal structure, localization of function, and derangements thereof has been the isolated nephron dissection technique developed by Oliver. It was felt that the usefulness of this method, particularly in regard to qualitative and quantitative localization of tubular function under various conditions, might be expanded through conjunctive use of autoradiography. The utility of autoradiography in the study of the reticuloendothelial functions of phagocytosis and pinocytosis is well documented. In this investigation, the somewhat analogous functions of athrocytosis and reabsorption by the renal tubular epithelium were analyzed. The application of this technique to the correlation of clinical and biochemical indices of functional derangement of the tubule with observed anomalies of radioautographic localization are discussed; e.g., uranium, aminonucleoside, immunologic nephroses, induced alterations of calcium and phosphorus excretion. Moreover, inferences regarding glomerular function may also be drawn, insofar as tubular epithelial components in certain loci reflected the content of the tubular lumen and hence the glomerular filtrate. As the specimen used in this method differed from histologic sections in terms of processing, integrity of cell membranes, etc., the advantages and the restrictions peculiar to this technique are stressed.

**HEMOCHROMATOSIS IN MEDITERRANEAN ANEMIA.** Camillus L. Witzleben and John P. Wyatt,\* St. Louis University School of Medicine, St. Louis, Mo.

Complete organ iron analyses, including the entire gastrointestinal tract, in several cases of "juvenile hemochromatosis" associated with Cooley's anemia were compared with those in idiopathic hemochromatosis and transfusional siderosis. The development of true cirrhosis in Mediterranean anemia as demonstrated clinically by a characteristic transition of the electrophoretically determined plasma protein pattern as well as pre- and post-mortem morphologic material are illustrated. Mechanisms contributing to iron surcharge in "juvenile" hemochromatosis will be discussed in the light of ferrokinetic data derived from radioisotopic studies in cases of thalassemia major.







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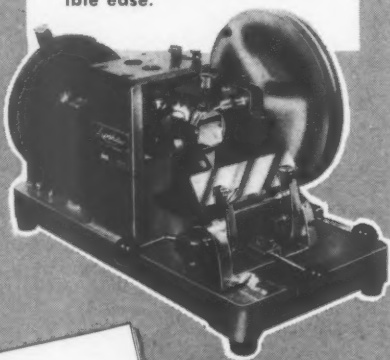
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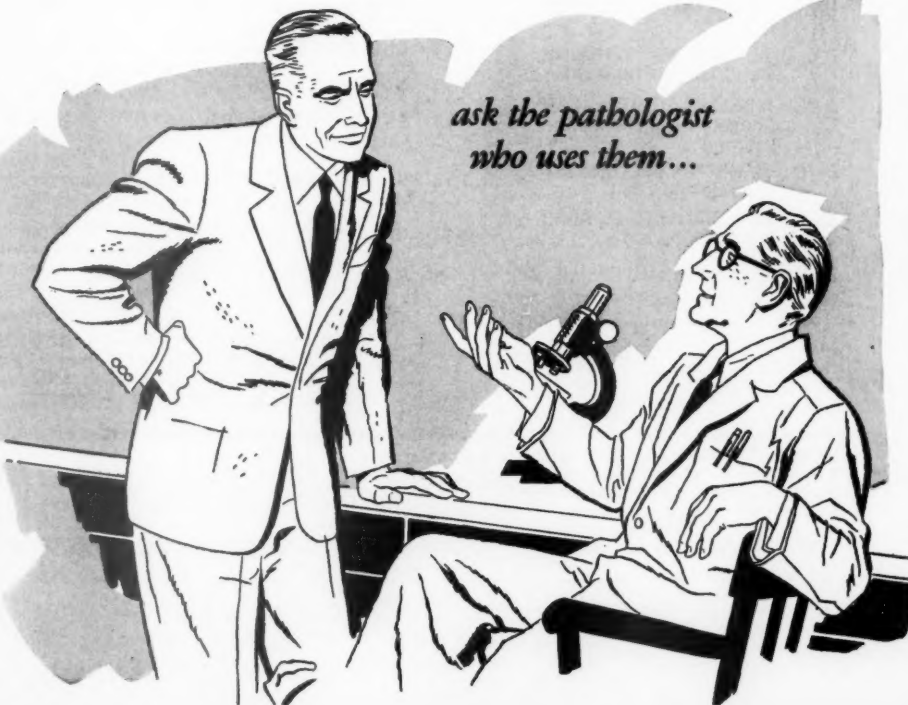


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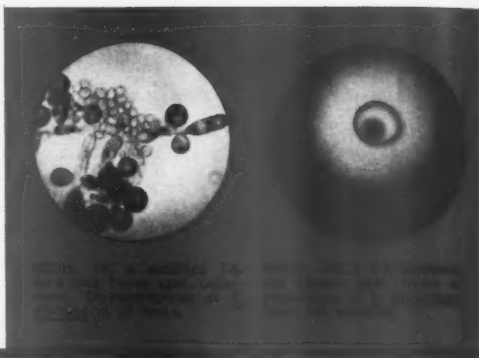
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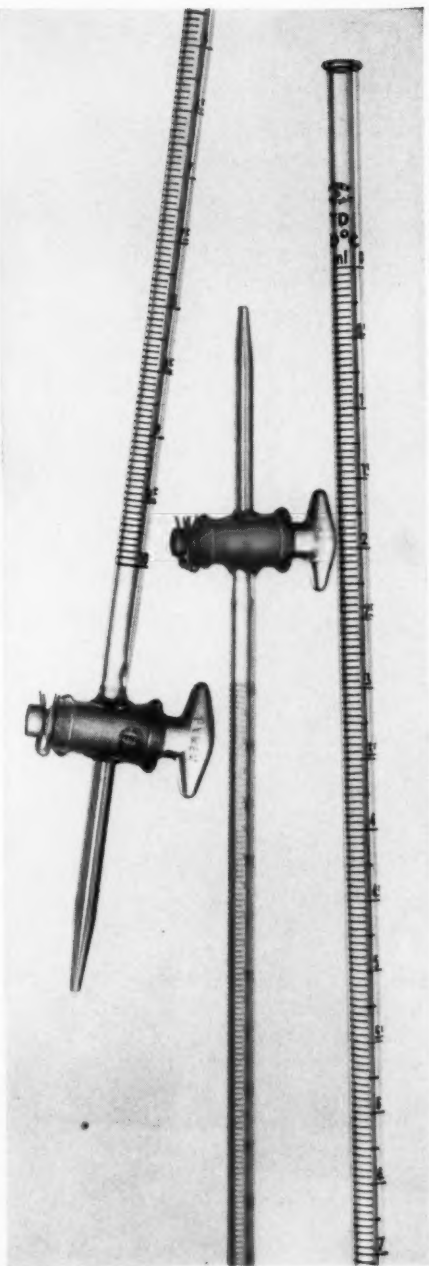
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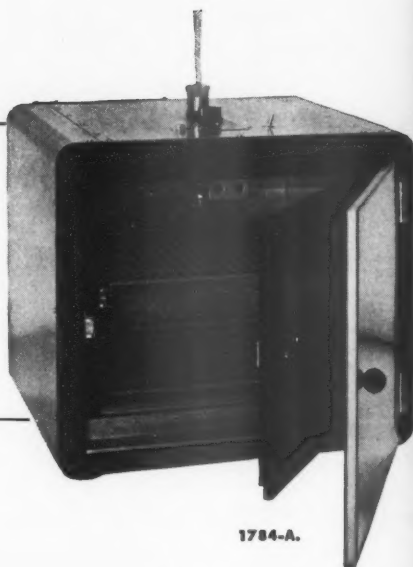
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